

**Mechanism of immune tolerance
induction in antigen-specific human
autoimmune disease**

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Abstract

Multiple sclerosis (MS) is an inflammatory disease that affects the central nervous system and is considered to be a T-cell mediated autoimmune disease. The “ideal” method in treating MS would be an antigen-specific therapy that does not require generalized immunosuppression. To date there are no definitive treatments for MS but there are several licensed therapies such as β -interferon. Unfortunately the effect of β -interferon (IFN β) is reduced by the development of neutralizing antibodies (NAbs) in up to 35% of MS patients within two years of starting treatment.

An immunization schedule was developed in the BALB/c mice by subcutaneous administration of recombinant human IFN β , and this resulted in development of high incidence of NAbs to the protein in the BALB/c model termed “NAbs model”. The mechanism of NAbs formation in this model is believed to be similar to that observed in IFN β -treated MS patients with NAbs, which is as a result of an immune response to the protein. We elected to study NAbs in the context of IFN β rather than MS directly to investigate the effects of antigen-specific tolerization strategies on the outcome of NAbs and indirectly on the outcome of IFN β treatment in MS disease.

The depletion of the immune cells triggers a reconstitution program that leads to renewal of the immune cell repertoire. Tolerance can be induced by intravenous administration of a protein. Within this window of reconstitution following depletion, it is hoped that the immune system can be manipulated to tolerate an otherwise foreign protein (human recombinant IFN β). The tolerance strategy employed in this project was immune cell depletion using antibodies and mitoxantrone, followed by intravenous re-introduction of rhIFN β . Tolerance was successfully induced in the NAbs model by intravenous administration of rhIFN β , and further enhanced by immune cell depletion prior to intravenous administration of rhIFN β .

The BALB/c “NAbs model” offers a suitable model for use in investigating induction of tolerance to rhIFN β following the formation of NAbs to the protein. The antigen of interest is known and the time to NAbs formation is also known. Tolerance induction can be monitored and investigated in this model.

Contents

Statement of originality	2
Abstract	3
List of Figures	8
List of tables	11
Abbreviations.....	12
Acknowledgements	15
1 Introduction	16
1.1 Multiple sclerosis	16
1.1.1 Clinical	18
1.1.2 Immunology and Pathology	19
1.1.3 Current treatments	21
1.2 Interferon-beta treatment.....	24
1.2.1 Anti-interferon-beta antibodies	25
1.2.2 Clinical and biological significance of anti-IFN β antibodies	26
1.3 Immunological tolerance	27
1.3.1 Central tolerance	28
1.3.2 Peripheral tolerance	30
1.3.3 Tolerance induction	32
1.4 Aims of this project	35
2 Materials and Methods.....	36
2.1 Animals	36
2.1.1 Biozzi ABH mice.....	36
2.1.2 BALB/c mice.....	36

2.2	Solutions and Media preparations.....	36
2.2.1	Complete RPMI culture medium	36
2.2.2	FACS Buffer	37
2.2.3	Lysis buffer	37
2.2.4	Complete NABs culture medium	37
2.2.5	Heat-inactivated IFN β (HI-IFN β)	38
2.3	Depletion reagents	38
2.4	Induction of neutralizing antibodies.....	38
2.4.1	Preparation of Interferon beta.	38
2.4.2	Preparation of Inoculum for NABs induction.....	39
2.4.3	Injection of animals.....	40
2.5	Induction of Experimental Allergic Encephalomyelitis.	40
2.5.1	Preparation of spinal cord homogenate	40
2.5.2	Preparation of inoculum for spinal-cord induced disease in ABH mice.....	41
2.5.3	Injection of animals.....	41
2.5.4	Clinical disease scoring	41
2.6	Tissue sampling	42
2.6.1	Blood sampling and analysis.....	42
2.6.2	Spleen sampling and analysis	42
2.7	Assessment of immune function	43
2.7.1	Surface antibody staining.....	43
2.7.2	Intracellular cytokine staining.....	44
2.7.3	CFSE labelling	45
2.7.4	Proliferation assay	46
2.7.5	Proliferation assay with cytokine production	46
2.8	Neutralizing antibody assay	47
2.8.1	Cell culture	47
2.8.2	Preparation of IFN β standard and samples	47
2.8.3	Incubation of samples with IFN β	49
2.8.4	Determination of the 50 % endpoint and Kawade correction factor.....	49
2.9	Statistical analysis.....	51
2.9.1	NABs assay	51
2.9.2	EAE Clinical score.....	51
2.9.3	Proliferation and cytokine assays	52

3	Establishment of neutralizing antibody model	53
3.1	Introduction	53
3.1.1	Development of antibodies to human Interferon beta	54
3.1.2	Establishment of neutralizing antibodies to human interferon beta in mouse models	56
3.2	Results	58
3.2.1	Induction of neutralizing antibodies in ABH mice	58
3.2.2	Induction of NAbs in BALB/c mice	71
3.3	Discussion	76
4	Development of depletion strategy	80
4.1	Introduction	80
4.1.1	Anti-CD4 antibody	80
4.1.2	Anti-CD20 antibody	81
4.1.3	Mitoxantrone	82
4.2	Results	84
4.2.1	Anti-CD4 depletion in BALB/c	84
4.2.2	Anti-CD20 antibody in BALB/c mice	87
4.2.3	Mitoxantrone in BALB/c mice	90
4.3	Discussion	94
5	Development of Tolerization Strategy	97
5.1	Introduction	97
5.1.1	Central tolerance	98
5.1.2	Peripheral tolerance	99
5.1.3	Tolerization strategies	101
5.2	Results	104
5.2.1	Induction of neutralizing antibody	104
5.2.2	Effects of intravenous recombinant human interferon on NAbs induction	106
5.2.3	Effects of depletion and tolerance induction strategies on NAbs	107
5.2.4	Proliferating cells and cytokine production	111
5.2.5	Effects of intravenous recombinant human interferon on proliferating T cells	116
5.2.6	Effects of depletion and tolerance induction strategies on proliferating T cells	123
5.3	Discussion	127
6	Experimental autoimmune encephalomyelitis and depletion strategy .	130

6.1	Introduction	130
6.1.1	Experimental autoimmune encephalomyelitis	130
6.1.2	Anti-CD4 antibody in EAE.....	132
6.1.3	Anti-CD20 antibody in EAE.....	132
6.1.4	Mitoxantrone in EAE	133
6.2	Results	134
6.2.1	Anti-CD20 antibody depletes B cells in the blood and spleen	134
6.2.2	B cell proportions similar in anti-CD20 treated and untreated mice following EAE induction ...	135
6.2.3	Acute anti-CD20 treatment does not alter EAE severity.....	137
6.2.4	Therapeutic anti-CD20 treatment does not alter EAE relapse	138
6.2.5	Multiple anti-CD20 treatment does not really alter EAE severity	140
6.2.6	Mitoxantrone and anti-CD4 antibody treatment alters EAE severity	141
6.3	Discussion	144
7	General discussion and conclusions.....	146
7.1	Key findings.....	146
7.2	Value of the NAbs mouse model	149
7.3	Future work	152
7.4	Conclusions	153
8	Reference List.....	154

List of Figures

Figure 2.1: EAE scoring scale.	42
Figure 2.2: Maximum and minimum LCPS and 50% endpoint.	50
Figure 3.1: Timeline for immunization schedule for the administration of interferon-beta in mice.	58
Figure 3.2: Neutralizing antibodies to Interferon-beta in ABH Biozzi mice.	59
Figure 3.3: Neutralizing antibodies to Interferon-beta in ABH Biozzi mice.	60
Figure 3.4: T cell responses to human Interferon-beta in ABH Biozzi mice.	62
Figure 3.5: Proliferative CD4 T cell responses to human Interferon-beta in ABH Biozzi mice.	63
Figure 3.6: Proliferative CD8 T cell responses to human Interferon-beta in ABH Biozzi mice.	63
Figure 3.7: Timeline for immunization schedule for the administration of interferon-beta in mice.	64
Figure 3.8: Neutralizing antibodies to Interferon-beta in ABH Biozzi mice.	65
Figure 3.9: Memory T cell responses to human Interferon-beta in ABH Biozzi mice.	67
Figure 3.10: Memory T cell responses to human Interferon-beta in ABH Biozzi mice.	68
Figure 3. 11: Memory T cell responses to human Interferon-beta in ABH Biozzi mice.	69
Figure 3.12: Memory T cell responses to human Interferon-beta in ABH Biozzi mice.	70
Figure 3.13: Timeline for immunization schedule for the administration of interferon-beta in mice.	71
Figure 3.14: CD8 T cell responses to human Interferon-beta in BALB/c mice.	73
Figure 3.15: Neutralizing antibodies to Interferon-beta in CFA immunized BALB/c mice.	75
Figure 4.1: CD4 T cell phenotyping in BALB/c mice following administration of anti-CD4 antibody.	85
Figure 4.2: Cell proportions in blood following administration of anti-CD4 antibody in naïve BALB/c mice.	86
Figure 4.3: Cell proportions in the spleen following administration of anti-CD4 antibody in naïve BALB/c mice.	87

Figure 4.4: Cell proportions in blood following administration of anti-CD20 antibody in naïve BALB/c mice.....	89
Figure 4.5: Cell proportions in spleen following administration of anti-CD20 antibody in naïve BALB/c mice.....	90
Figure 4.6: Cell proportions in the blood following administration of mitoxantrone in naïve BALB/c mice.	92
Figure 4.7: Cell proportions in the spleen following administration of mitoxantrone in naïve BALB/c mice.	93
Figure 5.1: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	105
Figure 5.2: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	105
Figure 5.3: Timeline for immunization schedule for the administration of interferon-beta, depletion strategy ad induction of tolerance in BALB/c mice.	108
Figure 5.4: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	109
Figure 5.5: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	110
Figure 5.6: Interferon-beta specific proliferation and interferon-gamma production in BALB/c mice.	115
Figure 5.7: Interferon-beta specific proliferation and interleukin-17 production in BALB/c mice.	115
Figure 5.8: CD4 T cell proliferation in BALB/c mice.....	116
Figure 5.9: Interferon-beta specific CD4 T cell proliferation and IFN γ production in BALB/c mice.	117
Figure 5.10: Interferon-beta specific proliferation in BALB/c mice.	118
Figure 5.11: Interferon-beta specific proliferation in BALB/c mice.	119
Figure 5.12: Interferon-beta specific T cell proliferation in BALB/c mice.	121
Figure 5.13: Interferon-beta specific T cell proliferation in BALB/c mice.	122
Figure 5.14: Interferon-beta specific T cell proliferation in BALB/c mice.	124
Figure 5.15: Interferon-beta specific T cell proliferation in BALB/c mice.	125
Figure 5.16: Interferon-beta specific T cell proliferation in BALB/c mice.	126
Figure 6.1: Percentage B cells in blood from ABH mice immunized intravenously with anti-CD20 antibody.	134
Figure 6.2: Percentage B cells in spleen of ABH mice immunized intravenously with anti-CD20 antibody.....	135
Figure 6.3: Timeline for immunization schedule for the administration of anti-CD20 antibody in EAE induced mice.....	136
Figure 6.4: Percentage B cells in blood of ABH mice post relapse EAE.	136

Figure 6.5: Treatment of relapsing remitting EAE with anti-CD20 antibody.....	137
Figure 6.6: Treatment of relapsing remitting EAE with anti-CD20 antibody.....	139
Figure 6.7: Treatment of relapsing remitting EAE with anti-CD20 antibody.....	140
Figure 6.8: Treatment of relapsing remitting EAE with anti-CD4 antibody or mitoxantrone.	142

List of tables

Table 2.1: Preparation of complete RPMI culture medium.....	36
Table 2.2: Preparation of lysis buffer.	37
Table 2.3: Preparation of NAb culture medium.	37
Table 2.4: Fluorochrome-conjugated antibodies used for labeling cells analyzed by flow cytometry.	44
Table 2.5: Preparation of antigen for stimulating cells.	45
Table 2.6: Preparation of IFN β stock solution.....	48
Table 2.7: Preparation of IFN β Standard curve.....	48
Table 2.8: Preparation of plasma and serum samples.	48
Table 3.1: Neutralizing antibodies to Interferon-beta in ABH Biozzi mice following immunization with Betaferon or Rebif.	65
Table 3.2: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	72
Table 3.3: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	74
Table 5.1: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	106
Table 5.2: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	107
Table 5.3: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	109
Table 5.4: Neutralizing antibodies to Interferon-beta in BALB/c mice.....	111
Table 5.5: Summary of Cytokine responses observed following different immunization schedules.....	112
Table 6.1: Treatment of relapsing remitting EAE with anti-CD20 antibody.....	138
Table 6.2: Treatment of relapsing remitting EAE with anti-CD20 antibody.....	139
Table 6. 3: Treatment of relapsing remitting EAE with anti-CD20 antibody.....	141
Table 6.4: Treatment of relapsing remitting EAE with anti-CD4 antibody or mitoxantrone.	143

Abbreviations

$\alpha\beta$	alpha-beta
ABH	Biozzi Antibody High mouse
APC	Antigen presenting cells
APL	Antigen peptide ligand
BAb	Binding antibody
BBB	Blood brain barrier
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CHO	Chinese hamster ovary
CO ₂	Carbon dioxide
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDSS	(Kurtzke) Expanded disability status scale
ELISA	Enzyme linked immunosorbent assay
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECDI	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FoxP3	Foxhead transcription factor
GA	Glatiramer acetate
HAT	Hybri-Max media supplement
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HLA	Human leucocyte antigen
IFN	Interferon
IFN α	Interferon-alpha
IFN β	Interferon-beta
IFN γ	Interferon-gamma
IFNAR	Interferon receptor
Ig	Immunoglobulin

IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
LCPS	Luminescent counts per second
LU	Laboratory unit
MHC	Major histocompatibility complex
mAb	Monoclonal antibody
MAG	Myelin associated glycoprotein
MOG	Myelin oligodendrocyte glycoprotein
MBP	Myelin basic protein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MxA	Myxovirus resistance protein
n	Number
NAbs	Neutralizing antibodies
NK	Natural killer cells
OD	Optical density
PBS	Phosphate buffered saline
PE	Phycoerythrin
PLP	Proteolipid protein
PPMS	Primary progressive multiple sclerosis
RNA	Ribonucleic acid
RRMS	Relapsing-remitting multiple sclerosis
SCH	Spinal cord homogenate
SEM	Standard error of the mean
SPMS	Secondary progressive multiple sclerosis
Sub-cut	Subcutaneous
TCR	T cell receptor
TGF β	Tumour growth factor-beta
Th1	T helper 1
Th17	T helper 17

Th2	T helper 2
Th3	T helper 3
TMB	Tetra-methyl-benzidine
T regs	T regulatory cells
Tr1	Type 1 regulatory cells
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

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Chapter 1

Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic neurodegenerative disease and the major cause of neurological disability in young adults (Noseworthy *et al.*, 2000). The immune system is thought to be a key player in the progression of the disease (Bruck, 2005). The immunological attack is directed against the central nervous system (CNS), which is made up of the brain, spinal cord and the cranial nerves. MS is a major demyelinating disease which involves a breakdown of myelin (demyelination), the insulating sheath that surrounds nerve fibre extensions called axons, exposes these axons and thus affects the ability of neurons to transmit signals through the axon. MS affects about 2.5 million individuals worldwide with an average age of onset between 20 and 40 years (Compston *et al.*, 2002).

To date, allocating the cause of MS disease to a single element has been elusive. It is plausible that susceptibility to disease is as a result of several interacting factors including genetic factors, age and environmental factors such as lifestyle, climate and vitamin D (Hauser *et al.*, 2006). Several bacterial and viral triggers have been proposed as causes for MS; however, so far conclusive evidence is missing. One such candidate is Epstein-Barr virus (EBV), which has been linked to MS (Ascherio *et al.*, 2007; Jilek *et al.*, 2008). 100% of MS patients have been infected with EBV in comparison with 95.8% of age-matched healthy controls and the risk of developing MS is increased in individuals who develop infectious mononucleosis (acute EBV infection) (Giovannoni *et al.*, 2006). The MS risk associated with infectious mononucleosis appears to persist throughout life. It has been suggested that EBV

infected B cells infiltrate the brain resulting in damage that lead to the initiation of MS disease (Serafini *et al.*, 2007). These observations have not been reproduced (Peferoen *et al.*, 2009; Willis *et al.*, 2009) as a result the role of EBV in MS pathology remains to be ascertained.

There are geographic differences in the prevalence of MS; the incidence of MS is highest in temperate regions including North America, Northern Europe, Southern Australia and New Zealand, with a decrease closer to the equator which is coincidental with annual sunlight exposure (Hayes *et al.*, 1997). Exposure to sunlight and UV has an effect on vitamin D synthesis and vitamin D has been suggested to have a positive role in MS (Pierrot-Deseilligny, 2009). Vitamin D is thought to be an immunomodulatory agent and has its effects on HLA gene expression by interacting with HLA-DRB1 promoter region, this region is preserved in MS associated with DRB1*1501 allele (Ramagopalan *et al.*, 2009). There is an increasing incidence of MS in females, approximately two thirds are female (Reingold, 2002). Age may also play a role in incidence, exposure to high or low-risk areas before age of early adulthood appears to play a major role in the incidence of MS. Once the high- or low-risks has been established in early adulthood, migration appears to play a smaller role in incidence of MS (Waksman, 1995).

A genetic association exists between certain major histocompatibility complexes (MHC) and the risk of developing MS. An association has been reported between HLA-DR2 (DRB1*1501, DRB5*0101, DQB1*0602) class II antigens and MS in Caucasian populations of northern and central Europe (Chao *et al.*, 2008; Lincoln *et al.*, 2005; Olerup *et al.*, 1991; Spurkland *et al.*, 1991). HLA-DR2 carries a fourfold relative risk for northern European Caucasians (Oksenberg *et al.*, 1993).

DRB1*0401 and DRB1*0301 are overrepresented in MS patients from southern Europe (Fogdell-Hahn *et al.*, 2000). Genome-wide association studies have recently been carried out and have revealed a number of loci that may be associated with the disease including IL2 receptor (Alcina *et al.*, 2009) and IL7 receptor (Lundmark *et al.*, 2007) and now there have been over 150 non-HLA gene regions linked to susceptibility to MS. (Sawcer *et al.*, 2011). Genetics plays a role in disease as is seen in the incidence rate in monozygotic twins (~30%) in comparison with dizygotic twins (~5%) (Compston *et al.*, 2008). The incidence is relatively low implying that other factors may also be at play in susceptibility to the disease (Sadovnick *et al.*, 1993).

1.1.1 Clinical

The diagnosis of MS is usually clarified by the 'McDonald Criteria' which requires dissemination of the disease process in both time and space, and exclusion of other causes (McDonald *et al.*, 2001). Clinical diagnosis is made using magnetic resonance imaging (MRI) scan and typically requires two relapses due to lesions in separate locations and different times (Fisniku *et al.*, 2008). A definitive diagnosis can take months or even years to establish with confidence in some cases (McDonald *et al.*, 2001; Nielsen *et al.*, 2007).

Individuals with MS typically experience one of a number of clinical courses of the disease, which may be mild, moderate or severe. Approximately 15% of MS patients experience benign MS in which case the patient completely recovers from episodes of the disease; the disease does not progress with time and there is no permanent disability (Ramsaransing *et al.*, 2006). Primary progressive MS (PPMS) is characterized by slow progression from the onset of disease in approximately 15% of MS patients, with steady worsening of disability and usually clinically develops at a later age (Andersson *et al.*, 1999). The most common clinical course of MS is

relapsing-remitting MS which affects approximately 85% of patients. There are episodes of exacerbation of the disease (relapse) from which there is partial or total recovery and remission (Noseworthy et al., 2000). As part of a relapse the patient may develop new symptoms or a worsening of pre-existing symptoms. Due to accumulation of damage in the central nervous system, the disease deteriorates to secondary progressive MS (SPMS).

Various neurological symptoms are present in MS patients depending on the part of the central nervous system involved in the disease. Neurological symptoms include weakness in limbs, limb tremor, optical neuritis, pain and spasms (Hauser et al., 2006).

1.1.2 Immunology and Pathology

The heterogeneous and unpredictable nature of MS adds to the complexity of determining the sequence of events leading to the cause of MS disease (McFarland et al., 2007). MS appears to be a T cell-mediated disease and the immune system a key player in the progression of the disease (Bruck, 2005). The immunological attack is directed against the central nervous system (CNS) which is usually associated with blood:brain barrier (BBB) dysfunction resulting in infiltration of mononuclear cells. Inflammatory events ultimately lead to neurological damage including demyelination and axonal loss (Weiner, 2004).

The antigen involved in initiating MS disease remains to be determined. A typical response to an antigen is focused on one or more dominant epitopes. With epitope spreading, there is diversification of the previously dominant epitopes which involves previously subdominant epitopes now becoming more dominant in the immune responses elicited. A spread of multiple antigenic determinants has been reported in MS patients over the time course of the disease. Patients with

established disease recognize more myelin epitopes than patients with recent-onset disease (T cell responses in MS broadens with time) and is thus implicated in the ongoing pathogenic process (Davies et al., 2005). Due to evidence of the involvement of myelin proteins in MS, altered peptide ligands (APL) of these proteins have been investigated as potential therapy in the disease. APLs are analogs of immunogenic peptides in which there have been changes in the T cell receptor contact sites. Some trials with APL have shown the potential of APL in MS therapy (Bielekova et al., 2000) but other trials have seen exacerbation of disease (Martin et al., 2001; Vergelli et al., 1996), and might even suggest that myelin may be the autoantigen in MS.

The factor or antigen and mechanism involved in MS is elusive but once initiated, it appears to activate autoreactive T cells in the periphery and leads to dysfunction of the BBB. Evidence of BBB disruption has been shown using gadolinium enhanced MRI (Alnemri et al., 1996). Adhesion molecules on the surface of activated T cells such as very late activation molecule-4 (VLA-4/CD49d) bind to its ligand vascular cell adhesion molecule-1 found on brain epithelium (Hemler, 1990) and this enables T cells cross the BBB. It is thought that once these autoreactive T cells (CD4+) cross the BBB and come in contact with autoantigens, an inflammatory cascade of events is initiated. T cell and antibody-mediated responses to various myelin proteins and activated membrane attack complexes of complement in the CNS have been described (Trbojevic-Cepe et al., 1998). Some target antigens for these T cells include myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG) and α B crystallin (Pender et al., 2007; van Sechel et al., 1999). CD4+ T cell initiated inflammation leads to stimulation of B cells, other T cells, macrophages and microglia, with amplified responses to pro-inflammatory molecules such as

chemokines, cytokines and adhesion molecules (Hjelmström et al., 1998). These pro-inflammatory cytokines promote BBB dysfunction by increasing permeability of the BBB and recruitment of other immune cells including T cells (Th1, Th2 and Th17 CD4+ cells), CD8 T cells natural killer (NK) cells and macrophages, into the CNS. These activated cells form free radical species such as nitric oxide which have been shown to kill oligodendrocytes and motor neurons (Bishop et al., 2009). This complex array of immunological events leads to demyelination, spontaneous remyelination and loss of axons (Neumann, 2003). Loss of myelin (demyelination) and oligodendrocytes, termed lesions are as a result of infiltration immune cells into the CNS and is the pathological hallmark of MS. Lesions can be found in the brain stem, cerebellum, optical nerves and in the spinal cord (Noseworthy et al., 2000). Remyelination occurs spontaneously following loss of myelin, but the capacity is eventually overwhelmed (Franklin et al., 2008). Demyelination leads to exposure and loss of axons and thus affects neurotransmission (Bjartmar et al., 2003), leading to development of additional symptoms such as limb tremors, fatigue and pain (Compston et al., 2002).

1.1.3 Current treatments

To date there are no definitive treatments which cure MS, but there are several therapies (which generally act as immunomodulators or immunosuppressive agents) for reducing relapse rate and severity of clinical outcome of early disease (Confavreux et al., 2004). These include Interferon-beta, glatiramer acetate, mitoxantrone, corticosteroids and several monoclonal antibodies including natalizumab (an anti-VLA4 monoclonal antibody). Some of these therapies however have adverse side-effects and are only moderately effective at reducing severity of disease outcome (Thrower, 2009).

Glatiramer acetate (Copaxone™, Teva Pharmaceuticals) is a synthetic amino acid copolymer of L-alanine, L-glutamic acid, L-lysine and L-tyrosine, an analogue for myelin basic protein (MBP). It reduces the frequency of neurological attacks when used in the treatment of relapsing-remitting MS (Khan *et al.*, 2001). The mode of action remains unclear but it is thought that Glatiramer acetate acts as an altered peptide ligand (APL) as it may cross-react with MBP and other myelin proteins and is thought to inhibit MBP-specific T cells, which may be important in MS (Dhib-Jalbut, 2003).

Corticosteroids are currently being used to treat relapses in MS patients. They are immunosuppressive in action, they alter the expression of adhesion molecules and vascular permeability (Tischner *et al.*, 2007). Steroids have been shown to speed up recovery from a relapse or disability (Brusaferri *et al.*, 2000). However, long term use of steroids has side effects and has moderate effect on relapse rate or outcome of disease progression. (Miller *et al.*, 2000).

Mitoxantrone (Novantrone®, Merck-Serono) is an anthracenedione which acts by intercalating into the DNA molecule, it also interferes with RNA, and uncoiling and repair of damaged DNA by inhibiting topoisomerase II enzyme (Fox, 2006). Mitoxantrone acts in a dose-dependent manner and is a potent immunosuppressive agent which inhibits in vivo induced proliferative response (Baker *et al.*, 1992). Clinical trials data suggest efficacy of mitoxantrone in reducing relapses and slowing disease progression in relapsing-remitting and secondary progressive MS (Debouverie *et al.*, 2007; Le Page *et al.*, 2008). The action of mitoxantrone is by non-specific immunosuppression of the immune system and cardiotoxicity is one of its serious side effects (Ghalie *et al.*, 2002).

FTY720 (Fingolimod, Gilenya™) is a sphingosine-1-phosphate receptor antagonist and inhibits the migration of lymphocytes from secondary lymphoid organs to the periphery (Brinkmann *et al.*, 2004). Fingolimod has been shown to reduce relapse rate and the risk of disability progression in RRMS (Kappos *et al.*, 2010). As with some other immunosuppressive agents, Fingolimod has several side effects including increased risk of infection by opportunistic bacteria/viruses and development of tumours (Cohen *et al.*, 2011).

Cladribine (Movectro™) is a purine nucleoside analogue. Its activated form, Cladribine triphosphate, disrupts DNA synthesis and repair and causes accumulation of deoxynucleotides (Brousil *et al.*, 2006). Initial clinical trials of Cladribine showed a significant reduction in relapse rates (Giovannoni *et al.*, 2010), however the drug has now been withdrawn from the market.

Several monoclonal antibodies (mAb) are being developed or are currently used in the treatment of MS. Natalizumab (Tysabri™) is a humanized monoclonal antibody (mAb) that binds to $\alpha 4$ chain of the $\alpha 4\beta 1$ integrin or very-late-antigen 4 (VLA4) which is expressed on all leucocytes except neutrophils. VLA binds to vascular cell adhesion molecule (VCAM) which is found on and upregulated in endothelial cells during inflammation (Luster *et al.*, 2005). Binding of Natalizumab® to VLA4 blocks it binding to VCAM thereby inhibiting the extravasation of leukocytes across the endothelium to target organs (Johnson, 2007). Natalizumab® was the first mAb to be approved in Europe and the USA for the treatment of relapsing-remitting MS (Miller *et al.*, 2003).

Alemtuzumab® (CAMPATH-1H) is a humanized mAb that binds to CD52 which is expressed on a number of cells of the immune system; binding results in the

depletion of the targeted cells (Moreau *et al.*, 1994). Alemtuzumab® therapy results in an impressive reduction in MS exacerbation, but consistent with effects of other immunosuppressive agents, it fails to inhibit progressive MS (Coles *et al.*, 2008).

Rituximab® is a chimeric mouse-human mAb that is targeted against CD20 expressed on B cells. Rituximab® depletes a subpopulation of B cells (pre-B and mature B cells) without affecting antibody production and secretion (Petereit *et al.*, 2008). Rituximab® has been shown to reduce relapse rate by about 50% in relapsing-remitting MS (Bar-Or *et al.*, 2008).

1.2 Interferon-beta treatment

Type I interferons (IFNs) include IFN α , IFN β , IFN ω and IFN κ , and are produced by a number of cell types in response to viral infections (Malmgaard *et al.*, 2003); they are capable of limiting the replication and spread of viruses during an infection (Bogdan, 2000). IFNs signal through receptors found on many cell surfaces (Zhang *et al.*, 2008). The type I IFN receptor (IFNAR) is made up of two subunits IFNAR1 and IFNAR2 which are found on target cells; binding of IFN β to the receptor results in signalling through the Janus kinase to induce certain genes, the products of which makes the target cell resistant to viral replication (Muller *et al.*, 1994). Mx proteins (MxA and MxB) are induced by type I IFNs, upon stimulation they accumulate in the cytoplasm of cells; MxA has been shown to have anti-viral properties (Haller *et al.*, 2002).

Interferon-beta (IFN β) is a licensed immune modifying therapy; there are two forms: IFN β -1a and IFN β -1b. IFN β -1a (Avonex®, Rebif®) is produced in Chinese hamster ovary cells and its amino acid sequence is identical to that of the natural IFN β (Kagawa *et al.*, 1988a). IFN β -1b (Betaferon®) on the other hand is produced

in *Escherichia coli* and its sequence is slightly different to the natural sequence; the cysteine residue is substituted with serine at position 17 and it lacks methionine-1 (McCullagh *et al.*, 1983). As IFN β -1b is produced in *E. coli* it is non-glycosylated. The action of IFN β in MS is unknown. Although IFNs have anti-viral activity, it is thought that IFN β acts on immune cells by modulating their actions via cytokine production, skewing their response from an IFN γ producing Th1 response towards a Th2 response (Hartung *et al.*, 2004). IFN β has the ability to reduce proliferation of lymphocytes by decreasing expression of activation markers such as IL2 receptors and IL10 an immunosuppressive cytokine, and thereby reduces the migration of activated inflammatory cells through the blood brain barrier (BBB) into the CNS (Noronha *et al.*, 1993; Rudick *et al.*, 1998). IFN β has been shown to reduce relapse rates in relapsing-remitting MS and prolong the time to confirmed progression in secondary progressive MS (Group, 1993).

1.2.1 Anti-interferon-beta antibodies

Naturally occurring proteins are usually tolerated by the body and these proteins do not generally invoke an immune response. When these naturally occurring proteins are synthesised or genetically modified, they may have changes made to their structure which may then lead to a loss of immune tolerance to the protein when administered. Due to the loss of tolerance to the protein, antibodies may be generated which are either binding antibodies (BAbs) or neutralizing antibodies (NAbs). Neutralizing antibodies generally bind to the protein and prevents the binding of the protein to its receptor and thus inhibits the action or bioavailability of the protein. BAbs do not significantly affect the action of proteins, but may act as carriers or may increase the elimination of the proteins.

Due to the difference in structure of IFN β -1a and IFN β -1b from the natural protein (Kagawa *et al.*, 1988b; McCullagh *et al.*, 1983), IFN β formulation, chronic administration of large amounts of protein and route of delivery, antibodies to these proteins develop over treatment time (Hartung *et al.*, 2007). Some IFN β -specific antibodies termed BAbs bind IFN β but do not significantly affect the binding of IFN β to its receptor or the activity of the protein. BAbs may form immune complexes with IFN β which are cleared from the circulation (Perini *et al.*, 2001). On the other hand, NAbs bind IFN β and reduce its bioavailability by blocking the binding of IFN β to its receptor mainly found on immune cells (Malucchi *et al.*, 2004).

Varying incidences of BAbs (10 to 90%) and NAbs (2 to 47%) have been reported in MS patients (Bertolotto *et al.*, 2004; Giovannoni *et al.*, 2002). Approximately 35% of MS patients develop NAbs to IFN β within 2 years of starting treatment; of these patients, approximately 50% will revert to a negative NAbs status. Persistently high positive titres of NAbs have been associated with a greater number of relapses or more progression in MS patients (Francis *et al.*, 2005; Sorensen *et al.*, 2003).

1.2.2 Clinical and biological significance of anti-IFN β antibodies

The clinical significance of NAbs to IFN β in MS patients is an ongoing debate and several studies have been done to investigate the significance on relapse rate and clinical outcome.

In a study comparing the efficacy of IFN β with Copaxone (BECOME Study), it was shown that patients with high levels of NAbs had diminished reduction in enhancing lesions on MRI and this was also associated with reduction in bioactivity of IFN β (Pachner *et al.*, 2009). Development of NAbs to Rebif in the PRISMS study led to a loss of significant reduction in relapse rate and MRI activity (PRISMS-4., 2001). In a longitudinal study, it was described that NAbs positive patients had fewer relapses

in the first 6 months of treatment when compared with NAb negative patients, but then after 6 months they experience higher relapse rates (Sorensen *et al.*, 2005).

There is a faster development of NAb in IFN β -1b-treated patients in comparison with IFN β -1a-treated patients; a higher protein dose of IFN β -1b is used by patients and may be responsible for this (Ross *et al.*, 2000). IFN β -1b is also a mutated protein, has reduced bioactivity and is non-glycosylated that may increase its immunogenicity. In a study, a higher proportion of NAb positive patients were observed in the high dose Avonex group and these patients had a higher relapse rate within 1-4 years of treatment (Kappos *et al.*, 2005).

NAb to IFN β appears to develop within 2 years of treatment but the clinical impact may not be felt until sometime later, this highlights the need for more long term follow-up for NAb positive patients. Over time, it has been shown that approximately 30% of NAb positive patients revert to a negative status mainly in patients with low NAb titres and those treated with IFN β -1b (Sorensen *et al.*, 2005).

1.3 Immunological tolerance

In a healthy immune system, discrimination between self and non-self-antigens usually occurs, resulting in a balance. This is termed immune tolerance which is the ability of the immune system to distinguish self from non-self and harmful from harmless molecules. Tolerance is also the inability to mount an immune response to a subsequent rechallenge with the same antigen. The route of antigen introduction is critical in determining the outcome of an immune response. Intravenous and mucosal administration of antigen for example, favours the induction of tolerance to the antigen (Verbeek *et al.*, 2007). Tolerance is a state of non-responsiveness in the presence of an antigen, in a healthy immune system these are generally self

antigens. There are two coordinated processes referred to as central and peripheral tolerance which results in functional tolerance to self-antigens. These processes include the deletion of autoreactive lymphocytes in the central lymphoid organs during lymphocyte maturation; and suppression of autoreactive lymphocytes in the periphery that may have escaped elimination.

1.3.1 Central tolerance

Central tolerance is the process by which lymphocytes reactive to self-antigen are largely eliminated during development in the primary lymphoid organs. The main method of elimination is by deletion through negative selection. In the case of T cells, central tolerance occurs when the bulk of self-reactive T cells are deleted during thymic development. T cell development occurs in the thymus, where rearrangement of the T cell receptor (TCR) genes occurs leading to the diversity of T cell population. T cells undergo TCR α - and β - locus rearrangement and are characterized as double positive (CD4+/CD8+). These double positive cells either survive or are eliminated depending on the interaction of the TCR with self-antigen-MHC complex. TCR rearrangement (receptor editing) may occur in these cells which may increase self-MHC restriction (McGargill *et al.*, 2000). A large proportion of these double positive cells do not display any specificity for a major histocompatibility complex (MHC) ligand and undergo apoptosis or "death by neglect" (Surh *et al.*, 1994). During intrathymic development, a small proportion of double positive T cells with TCR that bind with low avidity to antigen loaded in MHC molecules results in the maturation to the single positive stage- CD4+/8- or CD4-/8+ (Starr *et al.*, 2003). T cells with TCR that bind to self-antigen loaded on MHC molecules undergo one of 2 selections. T cells with low to medium affinity for self-antigen receive survival signals that ligate the TCR and leads to survival of these T

cells and is termed positive selection (Klein *et al.*, 2009). Another selection process termed negative selection leads to apoptosis of T cells with TCR with strong affinity for self-antigen, strong enough to have the potential of being autoimmune disease mediators. These cells are eliminated by apoptosis in the absence of costimulation and are usually Fas-dependent (Kishimoto *et al.*, 1998). This negative selection process is aided by the transcription factor Aire (autoimmune regulator). Aire is an autoimmune regulator gene and facilitates the thymic expression of tissue specific antigens by thymic epithelial cells (Anderson *et al.*, 2011). Several studies have shown that Aire-expressing cells are involved in T cell generation and negative selection (Perry *et al.*, 2014). The generation of particular T cells (such as T reg) at specific times during T cell development prevents autoimmunity (Tanaka and Sakaguchi, 2015). Anomalies in T cell generation and negative selection, and an imbalance between T regulatory cells and self-reactive T cells can lead to or predispose to, autoimmune disease. The ideal outcome of these selection processes would be a T cell repertoire with TCRs that may bind to self-MHC molecules but are unresponsive to self-antigen (Griesemer *et al.*, 2010).

B cell development occurs in the bone marrow (Osmond, 1990). Primary development of B cells is defined by the re-arrangement and expression of antigen receptor composed of immunoglobulin (Ig) light chain and heavy chain genes. The arrangement of the B cell receptor confers a high degree of antigen specificity and this influences clonal selection (Riolink *et al.*, 1991). Rearrangement of the heavy chain occurs in pro-B cells where D and J elements are ligated resulting in functional heavy chain protein synthesis (Kitamura *et al.*, 1992). These pre-B cells then undergo light chain rearrangement and express IgM on their surface. The antigen receptors displayed on the surface of pre-B cells then interact with antigen leading to clonal selection (for activation or elimination). Self-reactive B cells are deleted in

the bone marrow by clonal deletion, receptor editing and anergy (Wardemann *et al.*, 2003). Some autoreactive B cells successfully rearrange their light chain resulting in altered specificity and survival (Tiegs *et al.*, 2011). The surviving B cells leave the bone marrow for the periphery; these B cells are immature and have IgM expressed on their cell surface. B cells that survive negative selection differentiate to become mature B cells (naive cells) that express IgD, IgM and other proteins such as B220 in mice. B220 is expressed in all stages of B cell development in the mouse. The final stages of development of B cells take place in the spleen (Loder *et al.*, 1999); B cells undergo antigen-dependent differentiation into antibody-secreting and/or memory B cells. Developing B cells that bind weakly to self-antigen survive through positive selection and their Ig undergoes rearrangement (Levine *et al.*, 2000).

1.3.2 Peripheral tolerance

It has been reported that some self-reactive T cells may escape into the periphery (Liu *et al.*, 1995). These “escaped” cells may be dangerous as they can potentially be recruited and activated resulting in an autoimmune response. To monitor such potentially dangerous cells, a mechanism termed peripheral tolerance is in place to prevent these cells from orchestrating an autoimmune attack (Kisielow *et al.*, 1988; Walker *et al.*, 2002). Clones of cells that respond to self-antigen are generally deleted preventing the proliferation of these cells in response to the antigen (McCaughy *et al.*, 2008). The key mechanisms that maintain tolerance in the periphery include clonal deletion, anergy, ignorance, and regulation. Most cells of the immune system require two signals for the activation of the cells; T cells for instance require a signal through the TCR as well as a second co-stimulatory activation signal(s). The absence of the second activation signal leads to the induction of cell death or anergy (a state of unresponsiveness to antigen) and robust tolerance (Chen, 2004). One important costimulatory signal in T cell

activation results from the interaction of CD28 receptor on T cells and its ligands (CD80 and CD86) on antigen presenting cells (APC) (Sharpe *et al.*, 2002). This interaction leads to activation of T cells and anti-CD28 antibody was used in this project as a co-stimulatory molecule for cytokine production. Another costimulatory interaction is the binding of CD154 on activated T cells and CD40 on APCs (Croft, 2003). Blocking of these costimulatory signals has been shown to induce tolerance in allografts in rodents. (Foster *et al.*, 2003; Honey *et al.*, 1999); and regulatory T cells may play a role in the establishment of early tolerance (Verbinnen *et al.*, 2008).

Cell activation is delicately counterbalanced by the production of regulatory cells some of which appear to be inducible in the periphery; these regulatory T cells (T regs), are thought to be central to peripheral tolerance through their suppressive function (Bach, 2003). T regs suppress several cell types including CD4+ helper cells, CD8+ cytotoxic T lymphocytes, antibody production by B cells and APC function (Eddahri *et al.*, 2006; Mempel *et al.*, 2006; Taams *et al.*, 2000). In a subset of T regs known as CD25^{high}-FoxP3⁺ T regs, there is an upregulation of forkhead transcription factor (Foxp3) following activation, expression of FoxP3 does not always correlate with the suppressive function (Tran *et al.*, 2007). Another subset of T regs is the type 1 T regs (Tr1) that are suppressive through the production of high levels of IL10 and transforming grown factor beta (TGF β) (Groux *et al.*, 1997). Tr1 cells do not express Foxp3 (Vieira *et al.*, 2004). A third subset of T regs is known as Th3 cells, these are regulatory cells that are a result of conversion of naive CD4 T cells to inducible T regs by TGF β (Chen *et al.*, 2003). Of these 3 regulatory T cells, FoxP3⁺ T regs are the best studied and are thought to keep potentially autoreactive T cells unresponsive in the periphery (Sakaguchi *et al.*, 2008). Most T regs develop in the thymus with a few induced in the periphery from

naïve CD4 T cell pool (Fontenot *et al.*, 2005; Shevach *et al.*, 2008). T regs produced by the thymus have TCRs on their surface with increased affinity to self-antigen and this could be as a result of positive thymic selection for self-antigen or by clonal expansion of autoreactive clones in the periphery (Hsieh *et al.*, 2004). In addition, there are T regs reactive to foreign antigen and they expand and function in an antigen-dependent manner (Verma *et al.*, 2009). Tolerance interventions may boost the numbers and functions of T regs and/or inhibit the activation of naive T cells by co-stimulatory blockage (St Clair *et al.*, 2007).

1.3.3 Tolerance induction

A breakdown in tolerance can lead to an immune response to an antigen that would otherwise not elicit a response. A breakdown in tolerance to self-antigen for example is an underlying thread in many autoimmune diseases; and allergic diseases involve an impaired tolerance to otherwise harmless molecules. In MS, breaking of tolerance to auto-antigens e.g. myelin basic proteins, is thought to be of pathogenic relevance in the clinical outcome (such as lesion distribution) of the disease (Greer *et al.*, 2008). Induction of tolerance on the other hand is a deliberate process of inducing tolerance to an antigen where a response has been established or a response is likely. Many strategies for induction of tolerance have been reported in naive immune cells including oral and intravenous route of immunization and high or low dose antigen.

Oral therapies have been studied in the treatment of IgE-mediated food allergies such as milk, peanuts and eggs. Cells of the mucosa-associated lymphoid system play an important role in lymphocyte development and antigen presentation. The mucosa-associated lymphoid system continually undergo antigenic stimulation resulting mostly in oral tolerance rather than inflammatory immune reactions (Faria

et al., 2006). A breakdown of this immune responses or a shift of the immune responses from oral tolerance to immune reaction could lead to autoimmune or allergic diseases. Induction of oral tolerance is mainly achieved by prior oral administration of the protein which leads to suppression of humoral and cellular responses upon subsequent exposure to the protein (Faria *et al.*, 2006). Oral administration of antigens also leads to induction of regulatory T cells (Weiner *et al.*, 2011). One oral therapy for instance involves the ingestion of small amounts of the protein with increased doses over time leading to the exposure of the protein to tolerogenic APCs in the oral mucosa and these may potentially enhance the induction of tolerance to the protein (Allam *et al.*, 2008).

Intravenous administration of antigen has been shown to induce tolerance in naïve T cells by apoptosis or anergy; the major mechanism being antigen presentation without co-stimulation (Liblau *et al.*, 1996). Memory T cells have less stringent activation requirements than naive cells with reduced and in some cases no co-stimulation required, as such it can be difficult to induce tolerance in mice with memory T cells (Valujskikh, 2006). Tolerance has been successfully induced in memory T cells in mice by administration of soluble antigen (Hayashi *et al.*, 2002). Tolerance has also been induced by i.v. administration in established memory responses in haemophilia patients. Neutralizing antibodies to factor VIII (FVIII) develop over time in haemophilia A patients treated with FVIII products; this leads to inhibition of the products. Immune tolerance to FVIII has been achieved in these patients by continuous infusion of recombinant FVIII without this most haemophilia patients may have limited alternative treatment (Tamura *et al.*, 2006). In a mouse model of this condition, it was shown that high doses of i.v. FVIII led to the inhibition of FVIII-specific memory B cells (Hausl *et al.*, 2005). On the other hand, i.v. route of antigen introduction has been shown to boost antibody responses and i.v. MOG

stimulated a B cell response leading to the exacerbation of chronic EAE in marmosets (Genain *et al.*, 1996). In another study, the effect of the route of treatment with IFN β in a transgenic mice showed that i.v. treatment lead to the production of anti-IFN β antibodies and this was comparable with the i.m., i.p. and subcutaneous route of administration of the drug (Kijanka *et al.*, 2013). In a human study, IFN β was repeatedly given i.v. in NABs positive patients and this increased the bioavailability of the drug but did not appear to induce tolerance to IFN β (Hegen *et al.*, 2014). Antigen administration by i.v. has most consistently induced antigen-specific tolerance in established cell-mediated autoimmunity or hypersensitivity. The main mechanisms involved in i.v. induction of tolerance are clonal deletion, induction of regulatory T cells and anergy of antigen-specific T cells (Jacobs *et al.*, 1994). Variations to the i.v. method of tolerance induction have also been reported in animal models including cross-linking antigen (peptides) to splenocytes with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI) a cross-linking agent. Cross-linking of splenocytes with antigens induced an antigen-specific tolerance in the EAE model which led to prevention and treatment of disease in this model; tolerance was long-lasting and improved in comparison with soluble peptides (Turley *et al.*, 2007). Microparticles have also been used in prevention and treatment of EAE. Microparticles delays the release of antigens and this induced long-term T cell tolerance in mice with EAE, preventing the onset and modifying the course of the disease in these mice (Getts *et al.*, 2012). Another variation to i.v. tolerance induction is by the depletion of cell subsets prior to i.v. introduction of antigen. In the EAE model, it has previously been shown that reduction of cell subset pools (CD4 T cell depletion) prior to tolerance induction produced a robust unresponsiveness in established EAE disease (Pryce *et al.*, 2005).

1.4 Aims of this project

The aim of this project is to develop a neutralizing antibody “NABs” mouse model against recombinant human interferon beta for the study of tolerance induction, which is relevant for IFN β -treated multiple sclerosis patients that have developed neutralizing antibodies.

The objectives of the project are:

1. To develop neutralizing antibodies to recombinant human interferon beta in a mouse model that can be used to study the induction of tolerance (chapter 3).
2. To develop a strategy for the depletion of cell subsets in naïve mice and NABs mouse model using CD4+ and CD20+ depleting antibodies and mitoxantrone (chapter 4).
3. To develop a tolerization strategy in the NABs mouse model by intravenously re-introducing rhIFN β (chapter 5).
4. To further develop the tolerization strategy in the NABs mouse model by depletion of cell subsets prior to intravenous re-introduction rhIFN β (chapter 5).
5. To compare the effectiveness of depletion strategies in the well-established EAE model (chapter 6).

Achieving these objectives will result in the NABs model being used to study different tolerization strategies against the development of neutralizing antibodies to rhIFN β . These tolerization strategies can then be investigated in IFN β -treated MS patients with NABs to the protein.

Chapter 2

Materials and Methods

2.1 Animals

Mice were purchased and maintained on a 12h:12h light:dark cycle and received food and water *ad libitum*. All procedures were approved following ethical review processes in accordance with UK legislation (Animals (Scientific Procedures) Acts 1986).

2.1.1 Biozzi ABH mice

Adult male and female (6-8 weeks) Biozzi ABH mice were used and were purchased from Harlan UK Ltd (Bicester, UK). These mice were used for the EAE studies and preliminary NAbs studies.

2.1.2 BALB/c mice

Adult male and female (9-20 weeks) BALB/c mice were used and were purchased from Charles Rivers (Margate, Kent). These mice were used in most of the studies.

2.2 Solutions and Media preparations

2.2.1 Complete RPMI culture medium

Component	Final concentration	Volume (ml)
RPMI	-	450
Heat-inactivated FBS	10%	50
Penicillin	100 units	2.5
Streptomycin	100 µg	2.5
2- beta-mercaptoethanol	5 mM	5

Table 2.1: Preparation of complete RPMI culture medium. RPMI-1640 (Invitrogen, Paisley, UK), heat-inactivated foetal calf serum (Invitrogen, Paisley,

UK), 5 mM beta-mercaptoethanol (Sigma-Aldrich, Poole, UK) and penicillin/streptomycin (Sigma-Aldrich, Poole, UK).

2.2.2 FACS Buffer

Calcium and magnesium- free phosphate buffered saline (Invitrogen, Paisley, UK) was supplemented with 2% heat-inactivated foetal calf serum (Invitrogen, Paisley, UK).

2.2.3 Lysis buffer

Component	Concentration	Weight (g)	Volume (ml)
Ammonium chloride	15 mM	4.14	-
Potassium bicarbonate	10 mM	0.5	-
EDTA	20 mM	18.62	2.5
Distilled H ₂ O	-	-	500

Table 2.2: Preparation of lysis buffer. Ammonium chloride, Potassium bicarbonate, EDTA solution (Sigma-Aldrich, Poole, UK).

2.2.4 Complete NAb culture medium

Component	Volume (ml)
DMEM	500
FBS	57
Glutamine	6
7.5% w/v NaHCO₃ solution	6
HAT	1 vial
Complete culture medium	
Culture medium (above)	100
1 % Antibiotic/Antimycotic	1

Table 2.3: Preparation of NAb culture medium. DMEM, FCS (Invitrogen, Paisley, UK) glutamine, NaHCO₃, HAT and antibiotic-antimycotic solution (Sigma-Aldrich, Poole, UK).

2.2.5 Heat-inactivated IFN β (HI-IFN β)

1-10 $\mu\text{g/ml}$ working solution of recombinant IFN β (Betaferon® or Rebif®) in RPMI-1640 was incubated on a heating block at 75°C for 1.5 hours.

2.3 Depletion reagents

The reagents used for depleting cells in this project were anti-CD20 antibody (18B12, IgG2a; Biogen Idec, Cambridge, Massachusetts, USA), YTS191 CD4 depleting monoclonal antibody (Bio X Cell, West Lebanon, New Hampshire, USA) and mitoxantrone (Mayne Pharma, Warwickshire, UK).

- (1) 2.5 mg/ml of anti-CD20 antibody was prepared by adding 1 ml anti-CD20 antibody stock solution (5.67 mg) to 1.268 ml PBS. Animals were injected intravenously with 100 μl anti-CD20 antibody (250 $\mu\text{g}/\text{mouse}$).
- (2) 2 mg/ml of anti-CD4 antibody was prepared by adding 1 ml anti-CD4 antibody stock solution (10 mg) to 4 ml PBS. Animals were injected intraperitoneally with 100 μl anti-CD4 antibody (200 $\mu\text{g}/\text{mouse}$).
- (3) 625 $\mu\text{g/ml}$ of mitoxantrone was prepared by adding 1 ml mitoxantrone stock solution (2 mg) to 2.2 ml PBS. Animals were injected intraperitoneally with 100 μl mitoxantrone per 25 g of mouse (62.5 $\mu\text{g}/\text{mouse}$).

2.4 Induction of neutralizing antibodies.

2.4.1 Preparation of Interferon beta.

Interferon beta (Avonex®, Biogen-Idec; Betaferon®, Bayer Schering; or Rebif®, Merck-Serono) was diluted in PBS.

- (1) 667 µg/ml Avonex® was prepared by adding 1 mg Avonex to 1.5 ml PBS; this was further diluted 1:1 with CFA and animals injected sub-cut with 300 µl (100 µg Avonex per mouse).
- (2) 133 µg/ml Betaferon® was prepared by adding 1 mg Betaferon to 7.5 ml PBS; this was further diluted 1:1 with CFA and animals injected sub-cut with 300 µl (20 µg Betaferon per mouse).
- (3) 1.25 mg/ml Rebif® was prepared by adding 6.5 mg Rebif to 4.7 ml PBS. Animals were injected i.v. with 100 µl (125 µg Rebif per mouse).

2.4.2 Preparation of Inoculum for NABs induction.

A stock solution was prepared (Stock A) consisting of 4 ml incomplete Freund's adjuvant (Difco, Becton Dickinson, Oxford, UK), 16 mg Mycobacterium tuberculosis H37Ra and 2 mg Mycobacterium butyicum. This was stored at 4°C and discarded after a month. Mycobacterium stock was stored at -70°C. Complete Freund's adjuvant was prepared by adding 1 ml stock A to 11.5 ml incomplete Freund's adjuvant and vortex mixed before use. CFA is thought to enhance T cell responses and antibody production when used as an adjuvant in experimental models (Billiau & Matthys, 2001).

20 ml syringes (Becton Dickinson, Oxford, UK) were used to make up the inoculum solution. The plunger cap of the syringe was removed and the barrel was plugged with a stopper cap (Scientific Laboratory Supplies, Nottingham, UK). 5 ml diluted IFNβ solution (section 2.4.1) was added to 5 ml CFA in drops while vortexing to mix. The 20 ml syringe containing the solution was sonicated for 10 minutes in a

waterbath sonicator (Branson Ultrasonicator, Sigma, UK), this was to thicken the solution. The solution was then placed on ice to cool. A 1 ml syringe (Becton Dickinson, Oxford, UK) was inserted into the 20 ml syringe and the solution was pumped using the 1 ml syringe until it had thickened sufficiently that the solution did not disperse when a drop was added to water. The cap on the 20 ml syringe was removed and replaced with a 6 cm large bore needle and the solution dispensed into 1 ml syringes. A 16 mm 25 g needle (Becton Dickinson, Oxford, UK) was fixed to the 1 ml syringes and the needle cover pushed firmly onto the needles.

2.4.3 Injection of animals

Mice between 6-8 weeks were immunized. Mice were held at the nape of the neck and by the tail on a wire mouse cage. The skin of the dorsal surface of the flank was lifted and the needle inserted subcutaneously into the mouse. 150 μ l of IFN β solution was injected into the right flank and another 150 μ l was injected into the left flank. Mice were injected subcutaneously with various concentrations (5, 10, 20, 50, 70 or 100 μ g) of rhIFN β (Avonex®, Biogen-Idec; Betaferon®, Bayer Schering; or Rebif®, Merck-Serono). This was Day 0 and the process was repeated depending on the immunization protocol, and the injections were more posterior to the original injections.

2.5 Induction of Experimental Allergic Encephalomyelitis.

2.5.1 Preparation of spinal cord homogenate

Spinal cords from mice were removed and homogenised in a glass hand homogeniser and placed in -70°C freezer overnight. The homogenate was freeze dried for 24-48 hours in a freeze dryer (Edwards, Crawley, UK). The homogenate was ground to a fine powder and stored at -20°C.

2.5.2 Preparation of inoculum for spinal-cord induced disease in ABH mice

The procedure was followed as above in 2.4.2 except that 5 ml IFN β solution was replaced with 5 ml PBS containing 33 mg of freeze dried spinal cord homogenate (6.6 mg/ml).

2.5.3 Injection of animals

Mice between 6-8 weeks were immunized following the procedure in section 2.4.3 except that mice were immunized with spinal cord homogenates on Days 0 and 7 as previously described (Baker *et al.*, 1990). Acute hind-limb paralysis developed typically between 2-4 weeks after injection of SCH. A relapse could be induced 7-8 days after a further injection of spinal cord homogenates (O'Neill *et al.* 1991) and mice were immunized subcutaneously in the flank with SCH in Freund's adjuvant supplemented with 60 μ g Mycobacterium tuberculosis H37Ra and Mycobacterium butyricum on day 28-30.

2.5.4 Clinical disease scoring

Animals developing EAE were scored and weighed daily using a 5-point EAE scale (figure 2.1).

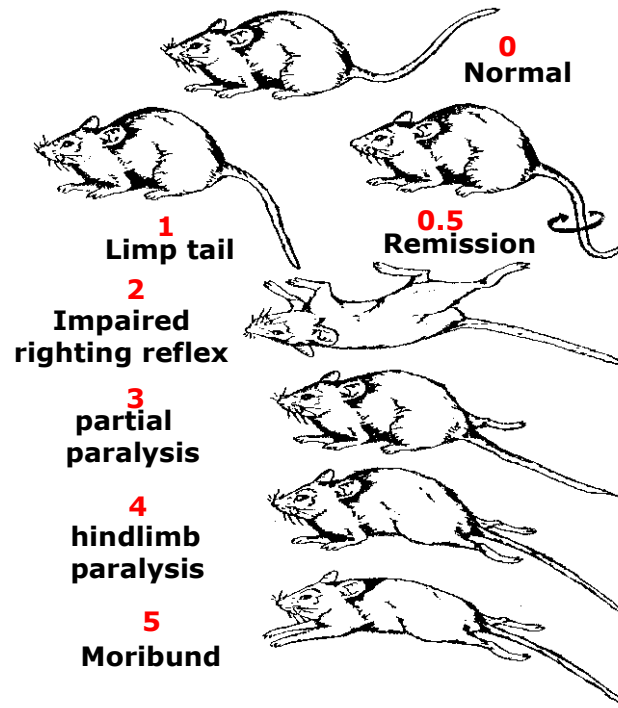


Figure 2.1: EAE scoring scale. EAE severity was scored following a 5-point scale. Paralytic clinical disease was scored daily: 0=normal, 1=limp tail, 2=impaired righting reflex, 3=hind-limb paresis, 4=complete hind-limb paralysis and 5=moribund/death; signs of reduced severity were scored at 0.5 less than the indicated grade as previously described (Baker et al., 2001).

2.6 Tissue sampling

2.6.1 Blood sampling and analysis

Cardiac or tail bleeds were collected in Eppendorff tubes containing heparin (Sigma-Aldrich, Poole, UK), samples were centrifuged and plasma/serum collected. Serum and plasma samples were taken from the mice prior to and post immunization.

2.6.2 Spleen sampling and analysis

Spleens were removed from dead mice killed by schedule 1 method and placed in PBS on ice or at 4°C. The spleens were forced through a 40 µm cell strainer into RPMI-1640 (Invitrogen, Paisley, UK) to form a cell suspension. Red cells were lysed by addition of 1 ml lysis buffer for a minimum of 1 minute at room temperature.

The number of viable cells was determined by trypan blue (Sigma-Aldrich, Poole, UK) exclusion method. Splenocytes were resuspended at $1-2 \times 10^6$ cells/ml in PBS.

2.7 Assessment of immune function

2.7.1 Surface antibody staining

1×10^6 cells/ml of splenocytes was stained with 1:10 dilution of surface antibodies (Table 2.4) in FACS buffer for 30 minutes in the dark. Cells were washed and fixed by addition of 100 μ l Caltag Medium A fixative (Caltag, Buckingham, UK) and incubated for minimum 15 minutes in the dark at room temperature. The cells were washed and resuspended in 300 μ l of FACS buffer and analysed by flow cytometry (Becton Dickinson, Oxford, UK).

Target	Fluorochrome	Clone	Manufacturer
CD4	FITC	YTS191.1	Serotec
F4/80	FITC	BM8	eBiosciences
CD4	PE	RM4-5	Caltag
IFN γ	PE	XMG1.2	BD Pharmingen
FoxP3	PE	FJK-16s	eBiosciences
IgG2a	PE	-	eBiosciences
CD25	PE-Cy7	PC61	BD Pharmingen
CD3	PE-Cy7	145-2C11	BD Pharmingen
CD8	PerCP	53-6.7	BD Pharmingen
CD4	APC	RM4-5	BD Pharmingen
CD45R/B220	APC	RA3-6B2	BD Pharmingen
Streptavidin	APC-Cy7	Streptavidin	BD Pharmingen
CD44	Biotin	IM7	BD Pharmingen
CD28	-	37.51	BD Pharmingen
CD49d	-	R1-2	BD Pharmingen

Table 2.4: Fluorochrome-conjugated antibodies used for labeling cells analyzed by flow cytometry. Fluorochrome abbreviations: FITC-fluorescein isothiocyanate, PE-phycoerythrin, PE-Cy7- tandem between Phycoerythrin and cyanine 5, PerCP-Peridinin-chlorophyll protein, APC-allophycocyanin, APC-Cy7- tandem between Allophycocyanin and cyanine 7.

2.7.2 Intracellular cytokine staining

1 ml aliquots were made by resuspending splenocytes from section 2.6.2 in complete culture medium at approximately $1\text{-}2 \times 10^6$ cells/ml (1×10^6 cells per test). Anti-CD28 (1 μ g) and anti-CD49d (1 μ g) antibodies (BD Pharmingen, Oxford, UK) were added to each tube and incubated for 10 minutes at room temperature. Cells were incubated with 1 ml of diluted antigen (table 2.5) or complete media per tube

for 1 hour at 37°C, 5% CO₂. Cells were further incubated with 10 µg of Brefeldin A (Sigma-Aldrich, Poole, UK) per tube overnight at 37°C humidified atmosphere containing 5% CO₂ (maximum of 18 hours incubation).

After overnight incubation, the cells were washed in FACS buffer and resuspended. Fluorochrome conjugated surface antibodies (table 2.4) were added and incubated for 30 minutes at room temperature in the dark and cells washed in FACS buffer. Cells were fixed by adding 100 µl of fixative reagent A (Becton Dickinson, Oxford, UK) and incubated for 15 minutes at room temperature in the dark. Cells were washed and 100 µl diluted fluorochrome conjugated anti-cytokine antibodies (table 2.4; antibodies diluted in permeabilizing buffer reagent B, Becton Dickinson, Oxford, UK) were added and incubated for 30 minutes at room temperature in the dark. The cells were washed and resuspended in 300 µl of FACS buffer and analysed by flow cytometry (Becton Dickinson, Oxford, UK). IFN β

Component	Initial concentration (/ml)	Final concentration (/ml)
IFN β	500 µg	10 µg
Heat-inactivated IFN β	500 µg	10 µg
Con-A	10 µg	1 µg
PMA	40 ng	20 ng
Ionomycin	1 µg	500 ng

Table 2.5: Preparation of antigen for stimulating cells. IFN β (Betaferon®, Bayer-Schering), Con-A, PMA and Ionomycin (Sigma-Aldrich, Poole, UK).

2.7.3 CFSE labelling

Splenocytes from section 2.6.2 were resuspended in RPMI-1640 at 2-10x10⁶ cells/ml. 5 µM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Sigma-Aldrich, Poole, UK) was added per ml of cell suspension and incubated for

10 minutes at 37°C. The reaction was stopped by addition of equal volume of FCS. The cells were then washed and resuspended at $1-4 \times 10^6$ cells/ml complete culture medium. Cells were used in sections 2.7.4 and 2.7.5.

2.7.4 Proliferation assay

Cells labelled with CFSE (section 2.7.3) were resuspended at $1-4 \times 10^6$ cells/ml in complete culture medium. 1 ml test aliquots (2×10^6 cells) were made and 1 ml diluted antigen (table 2.4) or complete culture medium was added to each aliquot and incubated for 3 days at 37°C humidified atmosphere containing 5%CO₂.

After incubation, the cells were washed and resuspended in FACS buffer. Fluorochrome conjugated surface antibodies (table 2.4) were added and incubated for 30 minutes at room temperature in the dark. The cells were washed and resuspended in FACS buffer. Cells were fixed by addition of 100 µl of fixative reagent A and incubated for 15 minutes at room temperature in the dark. The cells were washed and resuspended in 300 µl of FACS buffer and analysed by flow cytometry.

2.7.5 Proliferation assay with cytokine production

The process was followed in section 2.7.4 except that for the last 5 hours of the overnight incubation 20 ng Phorbol 12-myristate 3-acetate (PMA), 500 ng ionomycin and 5 µg Brefeldin A (Sigma-Aldrich, Poole, UK) was added to each test. Another exception was after incubating cells with fluorochrome conjugated surface antibodies and fixation, cells were further incubated for 30 minutes at room temperature in the dark with 100 µl diluted fluorochrome conjugated anti-cytokine antibodies (table 2.4; antibodies diluted in permeabilizing buffer reagent B). The cells were washed and resuspended in 300 µl of FACS buffer and analysed by flow cytometry.

2.8 Neutralizing antibody assay

2.8.1 Cell culture

The HL-116 cell line (Dr Gilles Uze, University of Montpellier, France) was used in this assay. This is a human fibrosarcoma cell line which has been stably transfected with firefly luciferase gene which is linked to the interferon stimulated response element. When IFN β binds to its receptor, the receptor cassette is activated and luciferase is released.

Frozen HL-116 cells were thawed and cultured in complete NAbs medium in 80 cm² culture flasks at 37°C, with 5% CO₂. Cells were sub-cultured when approximately 85% confluent when examined using a light microscope. Culture medium was discarded and 4 ml trypsin/EDTA (Sigma- Aldrich, Poole, UK) was added and incubated until cells were detached from culture flask. 1 ml FBS was added to inhibit the trypsin, and detached cells were aspirated and washed in PBS. Cells were counted using the trypan blue exclusion method and resuspended at 4x10⁵ cells/ml. 100 μ l of HL-116 cell suspension was added to each well of a 96 well plate (Greiner Bio-One, Stonehouse, UK) giving a final concentration of 4x10⁵ cells/well. Cells were incubated overnight at 37°C humidified atmosphere containing 5%CO₂.

2.8.2 Preparation of IFN β standard and samples

IFN β (Betaferon) was prepared by dissolving 1 vial in 16 ml NAbs culture medium to give a concentration of 1x10⁶ U/ml. This was serially diluted to give a working solution of 1000 U/ml (table 2.6).

IFN β (U/ml)	IFN β (ml)	Medium (ml)	Final IFN β (U/ml)
1,000,000	1	9	100,000
100,000	1	9	10,000
10,000	1	9	1,000

Table 2.6: Preparation of IFN β stock solution.

Interferon standard curve (100-3.125 U/ml) was prepared by serial dilution of stock solution (table 2.7).

IFN β (U/ml)	IFN β (ml)	Medium (ml)	Final IFN β (U/ml)
1000	1	4	200
200	4	4	100
100	2	2	50
100	4	16	20*
50	2	2	25
25	2	2	12.5
12.5	2	2	3.25
6.25	2	2	3.125
3.125	2	2	1.5625

Table 2.7: Preparation of IFN β Standard curve. 20* U/ml was added to each diluted sample.

Samples were diluted in NAbs culture medium (table 2.8)

Sample conc.	Sample (μ l)	Medium (μ l)	Dilution factor
Neat	15	285	1:20
1:20	100	100	1:40
1:40	150	150	1:80
1:80	150	150	1:160

Table 2.8: Preparation of plasma and serum samples.

2.8.3 Incubation of samples with IFN β .

150 μ l diluted samples (table 2.8) were incubated with 150 μ l diluted IFN β (table 2.7) for 1 hour at room temperature. Final concentration of IFN β was 10 U/ml. After incubation, 100 μ l/well of the sample/IFN β mixture was added to the HL-116 cells; IFN β standards were added in doubling dilutions to the cells and were incubated for 4 hours at 37°C humidified atmosphere containing 5%CO₂. The plates and the 'Promega Steady-Glo Luciferase Assay System' were brought to room temperature. The Promega steady-Glo (Promega, Southampton, UK) was added to each well (50 μ L) and incubated for 10 minutes at room temperature, and the plates were read on the Wallac Victor2 1420 Multilabel Counter Plate Reader set to read at luminescence.

2.8.4 Determination of the 50 % endpoint and Kawade correction factor

The Kawada technique defines the neutralization potency of serum as the titre that reduces IFN β bioactivity from 10 LU/ml to 1 LU/ml (ten-fold reduction in bioactivity of IFN β). The concentration of IFN β at the 50% endpoint is defined as 1 LU/ml, this is the point where 50% of the biological effect of IFN β is lost. A curve was constructed by plotting Log₁₀ IFN dilution (x-axis) vs. Log₁₀ LCPS (y-axis) to illustrate the dose-response of the cells to stimulation with IFN β . From these curves the maximal and minimal responses (anchor points) were identified and the 50% endpoint calculated. The minimal limit was set as the minimum amount of light the cells without any IFN β stimulus emit under the established conditions of the assay and the upper limit was taken as the maximum amount of light emitted by cells maximally stimulated by a concentration of IFN β that is considered to be saturating the transfected cell line.

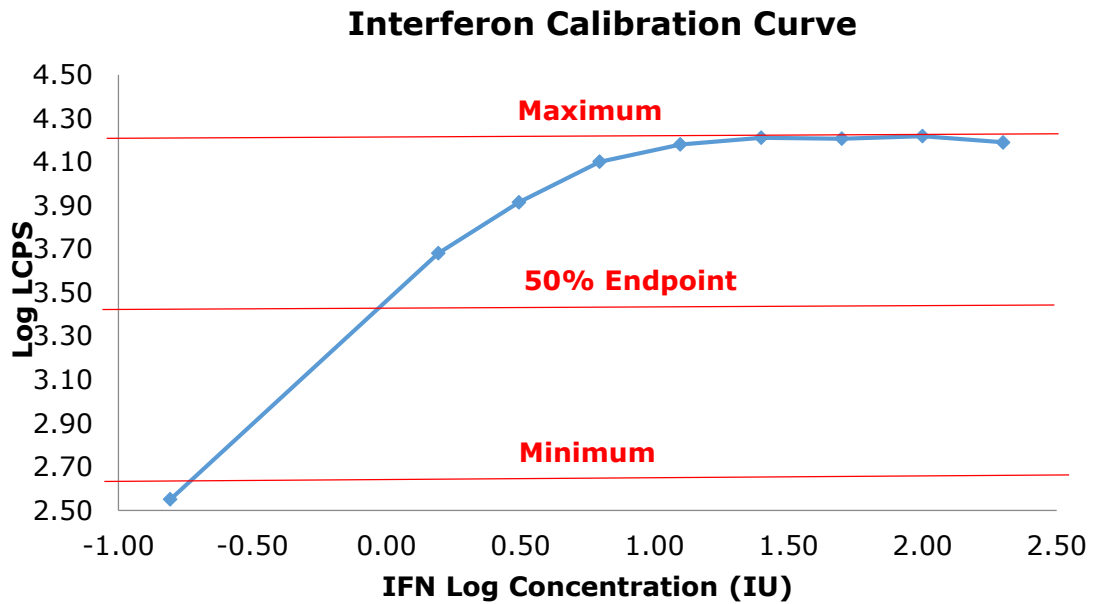


Figure 2.2: Maximum and minimum LCPS and 50% endpoint. LCPS is luciferase counts per second.

The concentration of IFN β at the 50% endpoint is defined as 1 LU/ml.

$$\text{50\% endpoint} = \text{Average (Log}_{10} \text{ Max LCPS and Log}_{10} \text{ Min LCPS)}$$

The concentration of IFN β at the 50% endpoint was calculated from the linear appearing part of the curve (figure 2.2) which was expanded and a trend-line fitted with an equation and R^2 value. This equation was used to calculate the Log IFN β dilution at the 50% endpoint (1 LU/ml). This was in turn used to determine the IFN β dilution (IFN β dilution = $10^{\text{Log IFN}\beta \text{ dilution}}$) and to calculate the LU/ml used to stimulate the cells in the assay.

$$n = \frac{\text{IFN}\beta \text{ dilution at 1 LU/ml}}{\text{IFN}\beta \text{ dilution used on cells}}$$

The value (n) was used in the Kawade formula as a correction factor. The ten-fold reduction (TRU/ml) in biological effect of IFN β was calculated using the formula:

$$t = \frac{f(n-1)}{9}$$

t is the titre, n is the number of LU/ml of IFN β applied to the samples, and f is the dilution of antibody at the 50% endpoint.

The 50% endpoint was also used to calculate the percentage inhibition for each sample tested.

$$\% \text{Inhibition} = \frac{(50\% \text{ endpoint} - \text{Sample LCPS})}{50\% \text{ endpoint}} \times 100$$

2.9 Statistical analysis

2.9.1 NAbs assay

Statistical analysis and graphs for the NAbs assays were prepared using Microsoft Office Excel (2007).

2.9.2 EAE Clinical score

The clinical score data is presented as the mean daily clinical score +/- standard error of the mean (SEM), the mean maximal clinical score of each group (group score) +/- SEM or the number of animals with EAE per group. Differences in clinical scores were assessed using non-parametric, Mann-Whitney U statistics (O'Neill *et al.*, 1993; Pryce *et al.*, 2003). Statistical analysis was performed by GraphPad Prism (GraphPad software, La Jolla, USA). Results were considered significantly different if the probability level $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) was reached between groups.

2.9.3 Proliferation and cytokine assays

Differences in cytokine production and proliferative responses were assessed by Students *t* test analysis using GraphPad Prism (GraphPad software, La Jolla, USA). Differences in cell proportions were assessed by Students *t* test analysis using GraphPad Prism. Results were considered significantly different if the probability level $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) was reached between groups.

Chapter 3

Establishment of neutralizing antibody model

3.1 Introduction

Interferon-beta (IFN β) is one of the first line treatments for multiple sclerosis (MS). Neutralizing antibodies (NAbs) to IFN β develop over treatment time and these affect the bioavailability of the drug. The aim of this chapter is to develop a neutralizing antibody model in the mouse similar to that observed in MS patients with NAbs to IFN β . This model can be used to study the effects of tolerization strategies on the outcome of NAbs to IFN β and indirectly on the outcome of IFN β treatment in MS.

We elected to study NAbs in the context of IFN β rather than MS directly to investigate the effects of tolerization strategies on the outcome of NAbs and indirectly on the outcome of IFN β treatment in MS disease. In MS, autoimmunity is unproven, the auto-antigens are not well defined and if the tolerization strategy goes wrong, it could exacerbate the disease (Vergelli *et al.*, 1996); while in NAbs positive patients on IFN β treatment, autoimmunity is proven, the auto-antigen and time course for developing antibodies is known and patients that already have a NAbs response are not physically unwell. The loss of endogenous IFN β activity as a result of NAbs does not cause disease but has been shown to reduce the availability of IFN β (Malucchi *et al.*, 2004).

Biozzi ABH mice and BALB/c mice were the 2 mouse models chosen to investigate the development of NAbs to human IFN β .

The ABH Biozzi mouse is used in experimental autoimmune encephalomyelitis (EAE) which is an autoimmune mouse model of MS; EAE can be induced by several CNS

peptides and by spinal cord homogenates in this model (Baker *et al.*, 1990). Biozzi ABH mice tend to produce a T helper1 (Th1) type immune response to foreign antigen and they produce mainly IgG1 antibodies (Baker *et al.*, 1990). This model was chosen as it is extensively used in our lab and by others as a model for MS.

BALB/c model is not routinely used as an EAE model, though it has been used to study the involvement of B cells in development of EAE (Lyons *et al.*, 2008). The BALB/c model is a T helper2 (Th2) biased model; Th2 T cells drive humoral immunity and allergic responses and produce IL-4 in an immune response. BALB/c has been used as a model to study a number of Th2-biased diseases including asthma. This model has been chosen as they produce antibodies readily in response to foreign antigen.

3.1.1 Development of antibodies to human Interferon beta

Interferons stimulate cellular responses to regulate viral infections, modulate the immune system and cell survival. Type 1 interferons have anti-viral activity and were developed for MS treatment as a result of this action (Borden *et al.*, 2007). The action of IFN β in MS is unknown. It is thought that IFN β acts on immune cells by modulating their actions via cytokine production skewing their response from an interferon-gamma (IFN γ) producing Th1 response towards a Th2 response (Hartung *et al.*, 2004). IFN β has the ability to reduce proliferation of lymphocytes by decreasing expression of activation markers such as IL2 receptors and IL10 an immunosuppressive cytokine, and thereby reduces the migration of activated inflammatory cells through the blood brain barrier (BBB) into the central nervous system (Noronha *et al.*, 1993; Rudick *et al.*, 1998). IFN β has been shown to reduce relapse rates in relapsing-remitting MS and prolong the time to confirmed progression in secondary progressive MS (Group, 2001).

Interferon is transiently produced in response to viruses and bacteria (Akira *et al.*, 2006). IFN β modulates immune cells by binding to IFN α/β receptors found on these cells (Zhang *et al.*, 2008). There are two forms of licensed IFN β for the treatment of MS: IFN β -1a and IFN β -1b. IFN β -1a (Avonex® and Rebif®) is produced in Chinese hamster ovary cells and its amino acid sequence is identical to that of the natural IFN β (Kagawa *et al.*, 1988b). IFN β -1b (Betaferon®) on the other hand is produced in *Escherichia coli* and its sequence is slightly different to the natural sequence; the cystine residue is substituted with serine at position 17 and it lacks methionine-1 and is non-glycosylated (McCullagh *et al.*, 1983). Antibodies to IFN β develop over treatment time (Hartung *et al.*, 2007); this could be due to the difference in structure of IFN β -1a and IFN β -1b from the natural protein (Kagawa *et al.*, 1988b; McCullagh *et al.*, 1983), IFN β formulation, chronic administration of large amounts of protein and route of delivery.

Some IFN β -specific antibodies termed binding antibodies (BAbs) bind IFN β but do not significantly affect the binding of IFN β to its receptor or the activity of the protein. BAbs may form immune complexes with IFN β which are cleared from the circulation (Perini *et al.*, 2001). Another group of IFN β -specific antibodies termed neutralizing antibodies (NAbs) can abrogate or neutralize the biological activity of IFN β by blocking the binding of IFN β to its receptor mainly found on immune cells (Malucchi *et al.*, 2004).

Varying frequencies of BAbs (10 to 90%) and NAbs (2 to 47%) have been reported in MS patients (Bertolotto *et al.*, 2004; Giovannoni *et al.*, 2004). Up to 35% of MS patients develop NAbs to IFN β within 2 years of starting treatment; of these patients, approximately 30% will revert to a NAbs negative status. Persistently high

positive titres of NABs have been associated with a greater number of relapses or more progression in MS patients (Francis *et al.*, 2005). There is a faster development of NABs in IFN β -1b-treated patients in comparison with IFN β -1a-treated patients; a higher protein dose of IFN β -1b is used by patients and may be responsible for this (Ross *et al.*, 2000).

3.1.2 Establishment of neutralizing antibodies to human interferon beta in mouse models

Interferon is produced in mice and in humans and there are differences in structure. Interferons mediate their effects by binding to the receptor on cells of the immune system; it has been shown previously that human cells are non-responsive to murine IFN β (Qin *et al.*, 2001). Administration of recombinant human IFN β (rhIFN β) will be seen by the mouse immune system as a foreign protein and an immune response should be mounted. The route of antigen introduction and the dose (high or low dose) is critical in determining the outcome of an immune response. Intravenous and mucosal administration of antigen for example, favours the induction of tolerance to the antigen. On the other hand subcutaneous and intraperitoneal administration of antigen favours an immune response mainly by antibody production.

P01575	IFNB_MOUSE 1	MNNRWILHAAFLLCFSTTALSINYKQLQLQERTNIRKCQELLEQLNGKINLTyr--ADFK	58
P01574	IFNB_HUMAN 1	MTNKCLLQIALLLCFSTTALSMSYNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFD	60
		* . *: **: *.***** :. *: * : : * :. :*:** **** ::: :	
		.*	

Table 3.1: Interferon beta amino acid sequence alignment between mouse and human. * signifies similar amino acid and : & . signifies different amino acids.

A transgenic mouse model resistant to human IFN β has been developed and used to study the breaking of tolerance to rhIFN β (van Beers *et al.*, 2011). The model has the human IFN β gene and sees the protein as “self” protein and will not normally mount a response to the protein. In transgenic mouse study, rhIFN β were oxidized and aggregated before immunizing these transgenic mice and these forms of IFN β broke tolerance and NAbs were observed in these mice. In the transgenic model, NAbs to rhIFN β were as a result of breaking of tolerance. In the BALB/c model used by group of Bellomi, administration of rhIFN β resulted in NAbs to the protein as the protein was seen as a foreign (Bellomi *et al.*, 2007). The transgenic mouse model is less suitable to predict immunogenicity of IFN β or to study tolerization strategies regarding NAbs formation as the mechanism of NAbs formation in this model is by the breaking of tolerance. The BALB/c NAbs model offers a more suitable model for use in investigating induction of tolerance to rhIFN β following the induction and formation of NAbs. The main mechanism of NAbs formation in the BALB/c model is similar to that observed in IFN β -treated MS patients with NAbs which is as a result of an immune response to the protein.

3.2 Results

3.2.1 Induction of neutralizing antibodies in ABH mice

Mice were immunized with recombinant human IFN β (Avonex®, Betaferon® or Rebif®) in CFA via the sub-cutaneous route (section 2.4); and sera/plasma samples collected (section 2.6) and tested in the neutralizing antibody assay (section 2.8). The 50% point between maximum and minimum log LCPS (luminescent counts per second) was determined and used to calculate the percentage inhibition; titres were also calculated for each sample tested (section 2.8.4). Naïve, unimmunized mice serum was used as a negative control.

Biozzi ABH mice (n=3) were immunized subcutaneously on days 0 and 7 with 100 μ g of rhIFN β -1a (Rebif®, Serono) in CFA per immunization (figure 3.1).

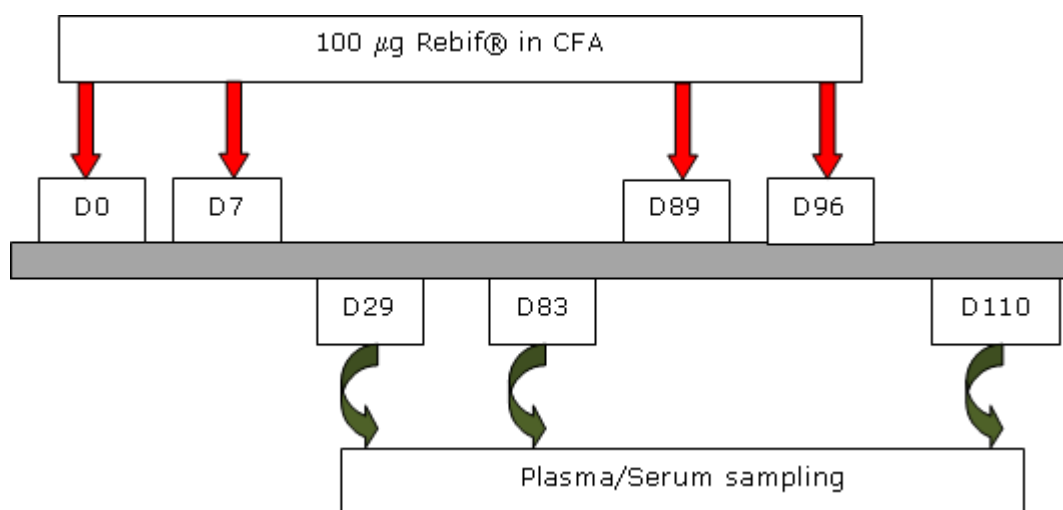


Figure 3.1: Timeline for immunization schedule for the administration of interferon-beta in mice. Biozzi ABH mice (n=3) were immunized with 100 μ g Rebif® in complete Freund's adjuvant on days 0, 7, 89 and 96. Plasma/Serum samples were taken and tested in the NAbs assay on Days 29, 83 and 110.

Serum samples were collected on day 29 and tested in the neutralizing antibodies (NAbs) assay. NAb were observed in these mice as shown in figure 3.2.

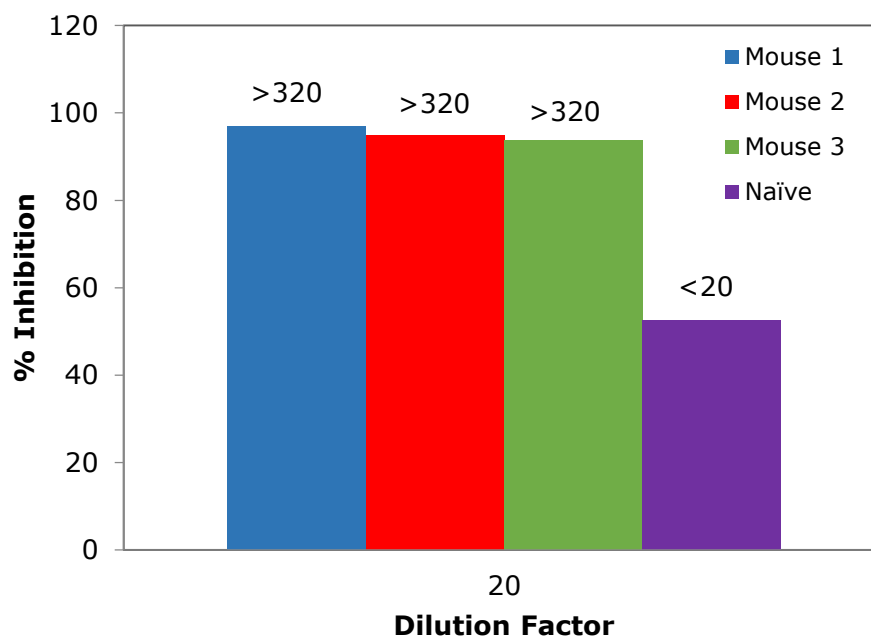
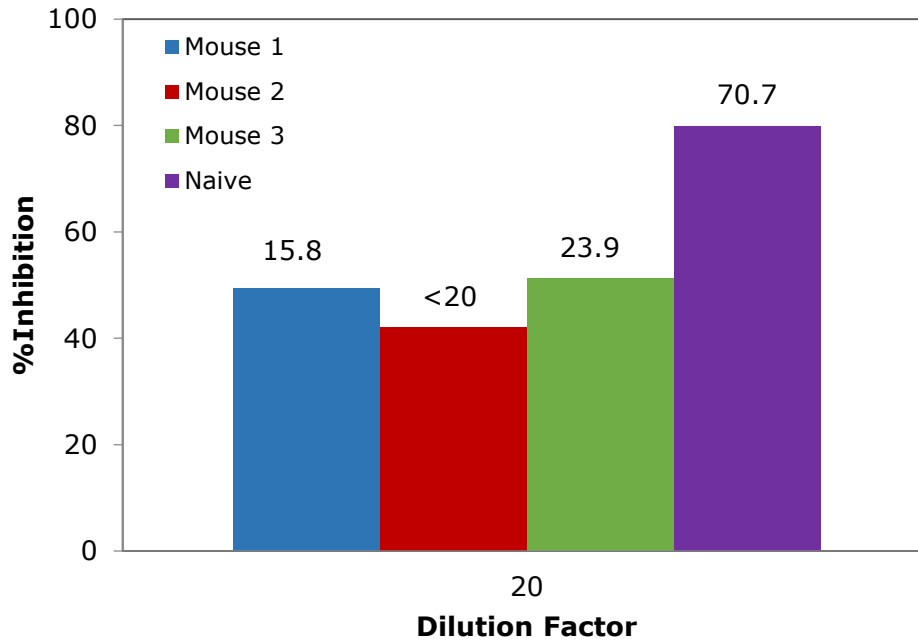


Figure 3.2: Neutralizing antibodies to Interferon-beta in ABH Biozzi mice. ABH mice (n=3) were immunized with 100 µg of rhIFNβ-1a (Rebif®) in complete Freund's adjuvant on days 0 and 7; plasma samples were tested in the NAbs assay on day 29. Naïve mouse serum was used as a control and was not immunized. %inhibition is the percentage of IFNβ that was inhibited by the sample upon incubation with IFNβ. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. NAbs to IFNβ were observed in all 3 test mice.

These mice were tested for NAbs on day 83 to investigate whether or not the NAbs observed post immunization were still detectable (figure 3.3a). No NAbs were observed in the test mice; the mice were further immunized with rhIFNβ (Rebif®) in CFA a third and fourth time (day 89 and 96) to see if NAbs could be re-induced in these mice. NAbs to IFNβ was observed in 1 of the test mice following these immunizations (figure 3.3b).

a)



b)

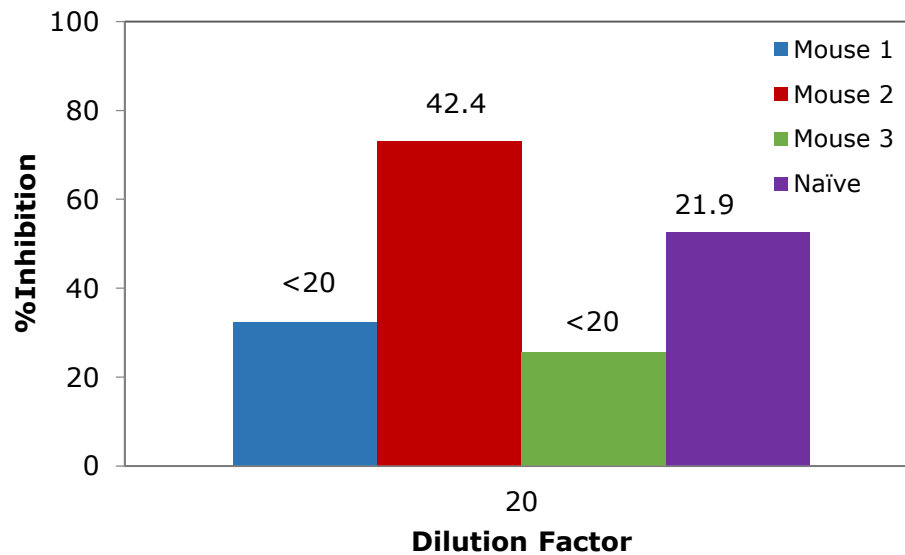


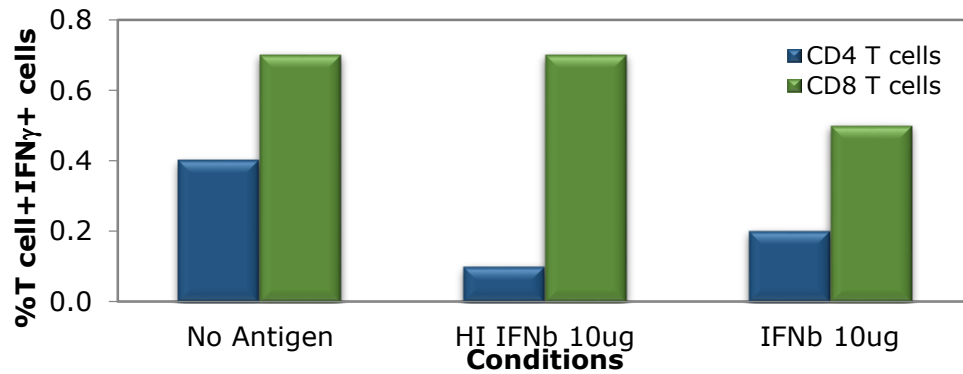
Figure 3.3: Neutralizing antibodies to Interferon-beta in ABH Biozzi mice.

ABH mice (n=3) were immunized with 100 μ g of rhIFN β -1a (Rebif®) in complete Freund's adjuvant on days 0, 7 (a), or days 0, 7, 89 and 96 (b); plasma samples were tested in the NAbs assay on days 83 (a) or 110 (b). Naïve mouse serum was used as a control and was not immunized. %inhibition is the percentage of IFN β that was inhibited by the sample upon incubation with IFN β . Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. No NAbs to IFN β was observed in all 3 test mice on day 83 (a) and only in 1 of the test mice on day 110 (b) had NAbs to rhIFN β .

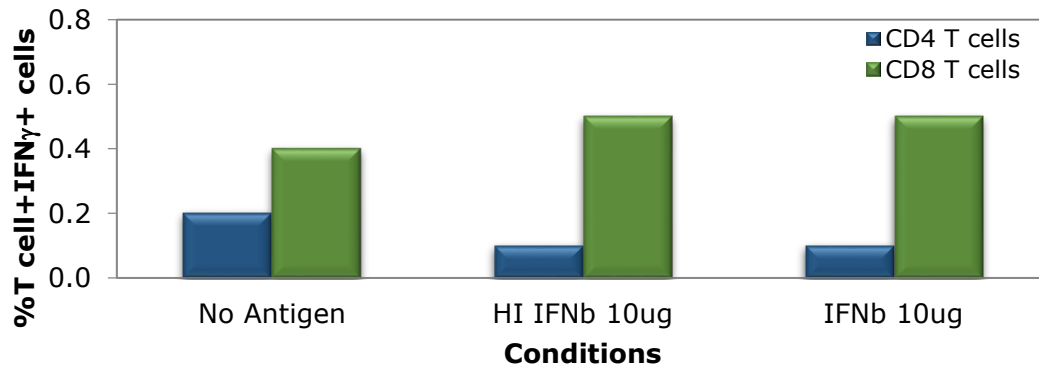
The terminal mice splenocytes were sampled in an intracellular cytokine assay (ICS) to investigate the cytokine response to recall antigen (rhIFN β). No significant increase in antigen-specific responses over the control responses was observed in both CD4 and CD8 T cells (figure 3.4).

Proliferative responses to human IFN β were investigated in the splenocytes at the terminal time point. Splenocytes were incubated for 3 days with CFSE (to investigate proliferative responses) and then cells were stained for CD4 and CD8 T cells. The control samples were not stimulated with IFN β (No Antigen). The percentage of proliferating CD4 or CD8 T cells producing IFN γ in response to human IFN β was calculated. A significant antigen-specific response to IFN β was observed in CD4 T cells in 1 mouse (Figure 3.5) and in 2 mice for CD8 T cell responses (Figure 3.6). The results observed here shows that though the samples at this time point may not have had detectable NAb, they had cytokine responses in response to presentation of human IFN β .

A)



B)



C)

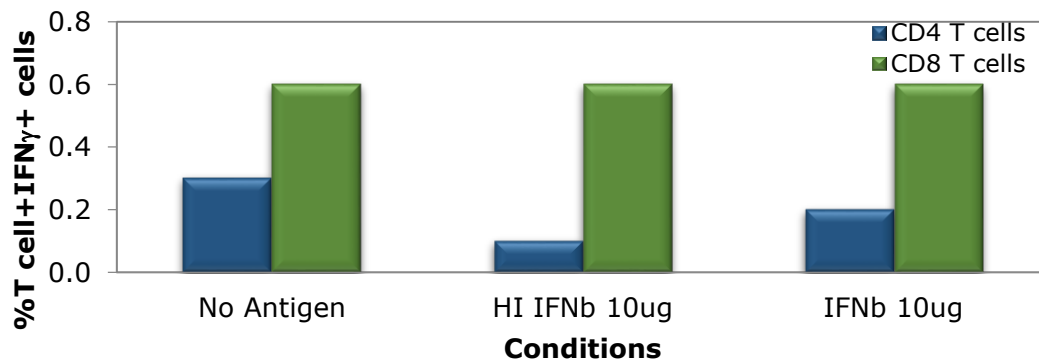


Figure 3.4: T cell responses to human Interferon-beta in ABH Biozzi mice.

ABH mice (n=3) were immunized with 100 μ g of rhIFN β -1a (Rebif®) in complete Freund's adjuvant on days 0, 7, 89 and 96; splenocytes were tested in the intracellular cytokine assay on day 110. No significant antigen-specific IFN γ responses by CD4 or CD8 T cell was observed in test mice over control responses (no antigen controls). Graphs A), B) and C) represents each mouse tested. HI IFNb is rhIFN β heated at 75°C for 1.5 hours.

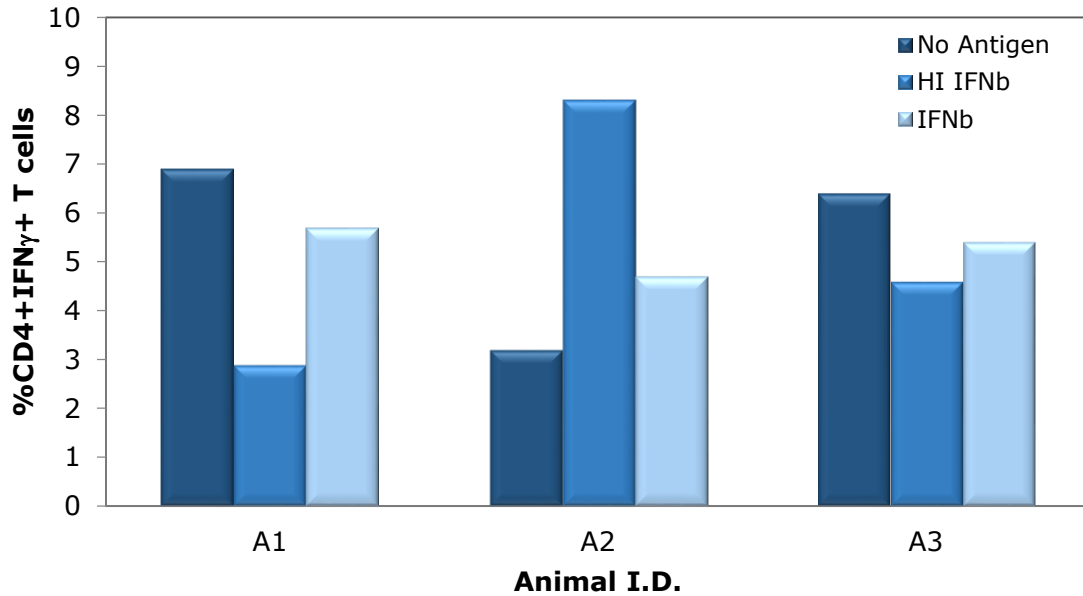


Figure 3.5: Proliferative CD4 T cell responses to human Interferon-beta in ABH Biozzi mice. ABH mice (n=3) were immunized with 100 μ g of rhIFN β -1a (Rebif®) in complete Freund's adjuvant on days 0, 7, 89 and 96; splenocytes were tested in the proliferation assay on day 110. Antigen-specific IFN γ response by CD4 T cells was observed in 1 test mice over control responses (no antigen controls).

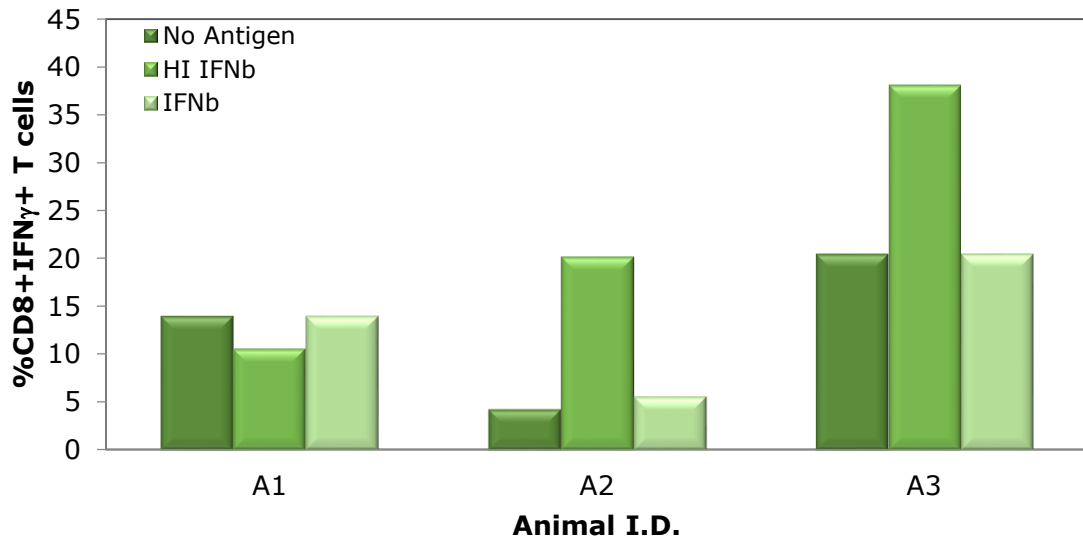


Figure 3.6: Proliferative CD8 T cell responses to human Interferon-beta in ABH Biozzi mice. ABH mice (n=3) were immunized with 100 μ g of rhIFN β -1a (Rebif®) in complete Freund's adjuvant on days 0, 7, 89 and 96; splenocytes were tested in the proliferation assay on day 110. A significant antigen-specific IFN γ response by CD8 T cells was observed in 2 test mice over control responses (no antigen controls).

Following this study and following discussions with a Statistician, the number of mice was increased in each group. ABH mice (n=30) were immunized subcutaneously on days 0, 7 and 21 with 5, 10 or 20 μ g of human IFN β in CFA per immunization (figure 3.7). The immunization schedule was changed to mimic the one routinely used in the lab for inducing EAE in the EAE mouse model.

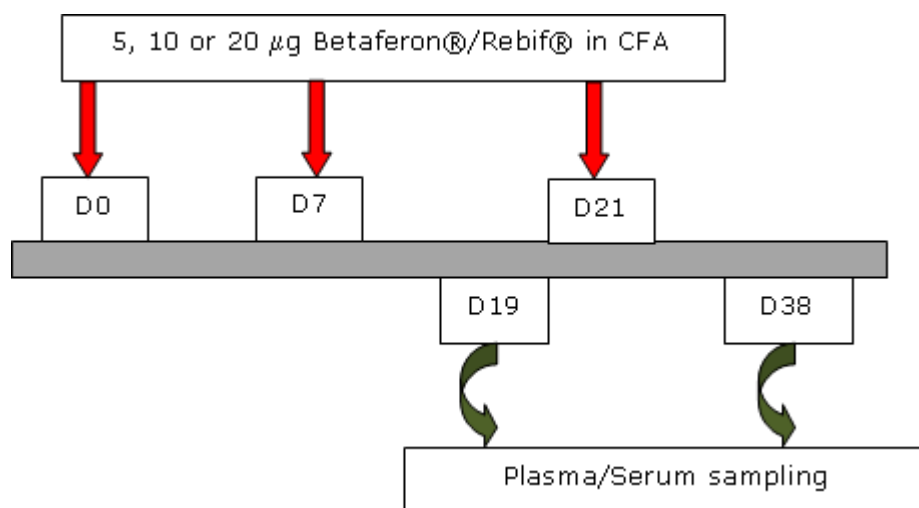


Figure 3.7: Timeline for immunization schedule for the administration of interferon-beta in mice. Biozzi ABH mice were immunized with 5, 10 or 20 μ g Rebif® (n=15) or 5, 10 or 20 μ g Betaferon® (n=15) in complete Freund's adjuvant on days 0, 7 and 21. Plasma/Serum samples were taken on Days 19 and 38.

Half (n=15) of the mice were given Rebif® new formulation (IFN β -1a) and the other half (n=15) were given Betaferon® (IFN β -1b). Plasma samples were taken on days 19 (pre-third immunization) and day 38 (2 weeks post third immunization) and tested in the NAbs assay. None of the samples were NAbs positive on day 19 (data not shown). On day 38, 33% of test mice (n=5) were NAbs positive following high dose Betaferon; on the other hand with Rebif, 80-100% of test mice were NAbs positive across all the doses given (figure 3.8 and table 3.1).

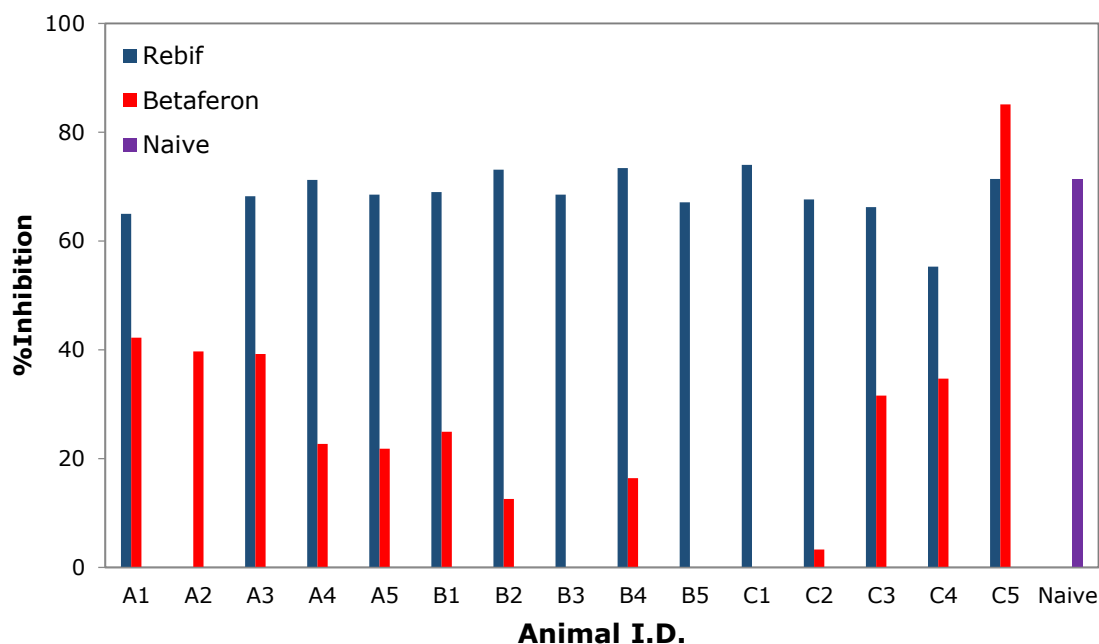


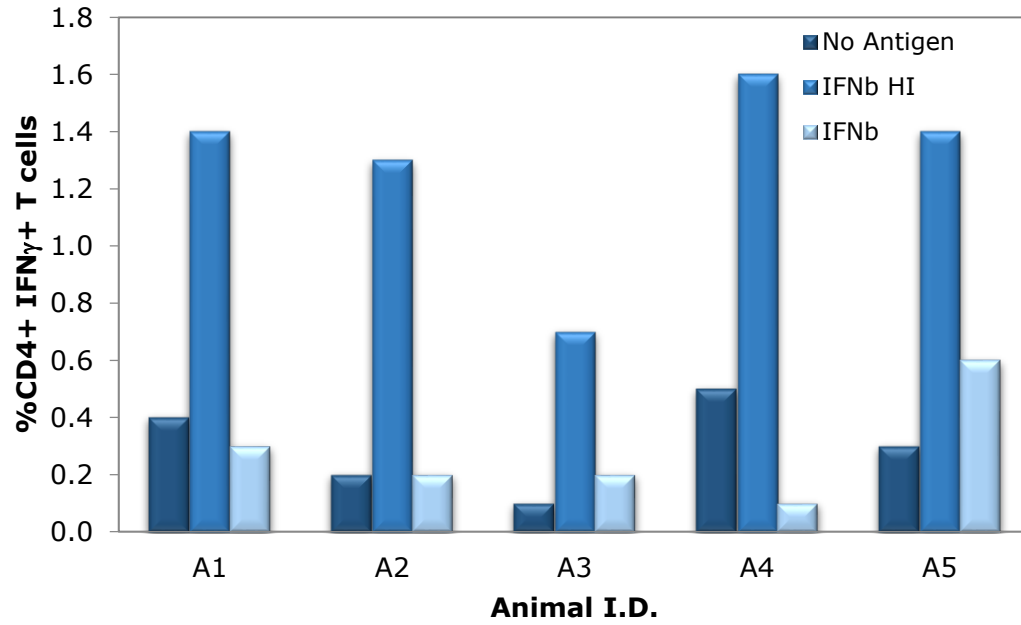
Figure 3.8: Neutralizing antibodies to Interferon-beta in ABH Biozzi mice. Biozzi ABH mice were immunized with 5, 10 or 20 µg Rebif® (n=15) or 5, 10 or 20 µg Betaferon® (n=15) in complete Freund's adjuvant on days 0, 7 and 21; plasma samples were tested in the NAbs assay on day 38. Naïve mouse serum (mouse 4) was used as a control and was not immunized. %inhibition is the percentage of IFNβ that was inhibited by the sample upon incubation. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. NAbs to IFNβ was observed in 18 of the 30 test mice.

	5 µg		10 µg		20 µg	
Product	Negative	Positive	Negative	Positive	Negative	Positive
Betaferon	5	-	5	-	2	3
	(<20)		(<20)		(<20)	(159)
Rebif	1	4	-	5	1	4
	(<20)	(>320)		(>320)	(<20)	(>320)

Table 3.2: Neutralizing antibodies to Interferon-beta in ABH Biozzi mice following immunization with Betaferon or Rebif.

Due to lack of consistent NAb induction in these mice with Betaferon, the amount of human IFN β was increased to see if that could induce detectable NAb in this model. Each group of mice (n=5) were given 20, 50, 70 or 100 μ g Betaferon® in CFA per immunization on days 0, 7 and 21. Plasma samples were taken on day 35 and tested in the NAb assay. Similar results were observed as was seen in figure 3.8 and table 3.1, even with an increase in the amount of rhIFN β given at immunization. The terminal splenocytes from mice immunized with Betaferon were sampled in an intracellular cytokine assay (ICS) to investigate the cytokine response in the memory cells to recall antigen (rhIFN β) detailed in section 2.7. An IFN β -specific response by memory CD4 and CD8 T cells were observed in all the mice immunized with 100 μ g Betaferon (figure 3.9) and 20 μ g Betaferon (figure 3.12). 4 out of 5 mice immunized with 70 μ g Betaferon (figure 3.10) and 50 μ g Betaferon (figure 3.11) had IFN β -specific IFN γ responses by memory CD4 and CD8 T cells. These results show that a memory cytokine response had developed over time, even though the levels of NAb were not detectable in these studies.

A)



B)

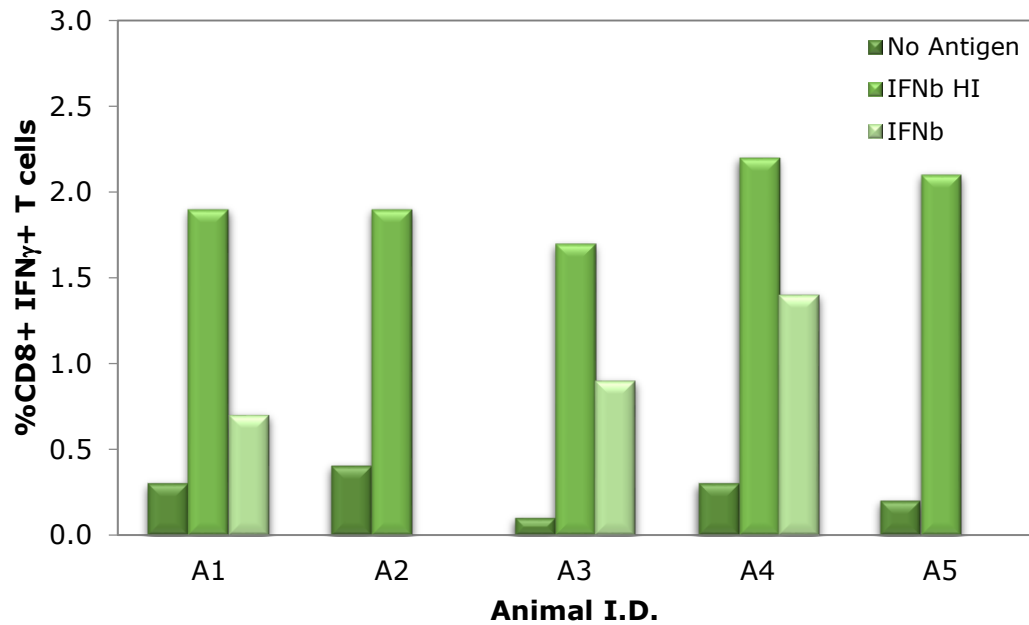
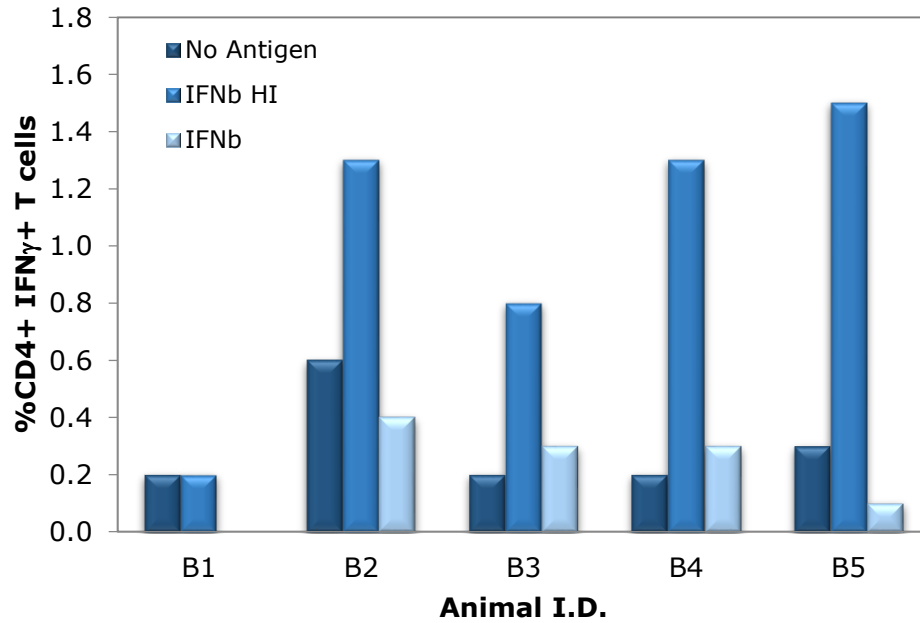


Figure 3.9: Memory T cell responses to human Interferon-beta in ABH Biozzi mice. Biozzi ABH mice were immunized with **100 μ g** Betaferon® (n=5) in complete Freund's adjuvant on days 0, 7 and 21; splenocytes were tested in the intracellular cytokine assay on day 38. An IFN β -specific IFN γ response by memory CD4 (A) and CD8 (b) T cells was observed in all test mice over control responses (no antigen controls).

A)



B)

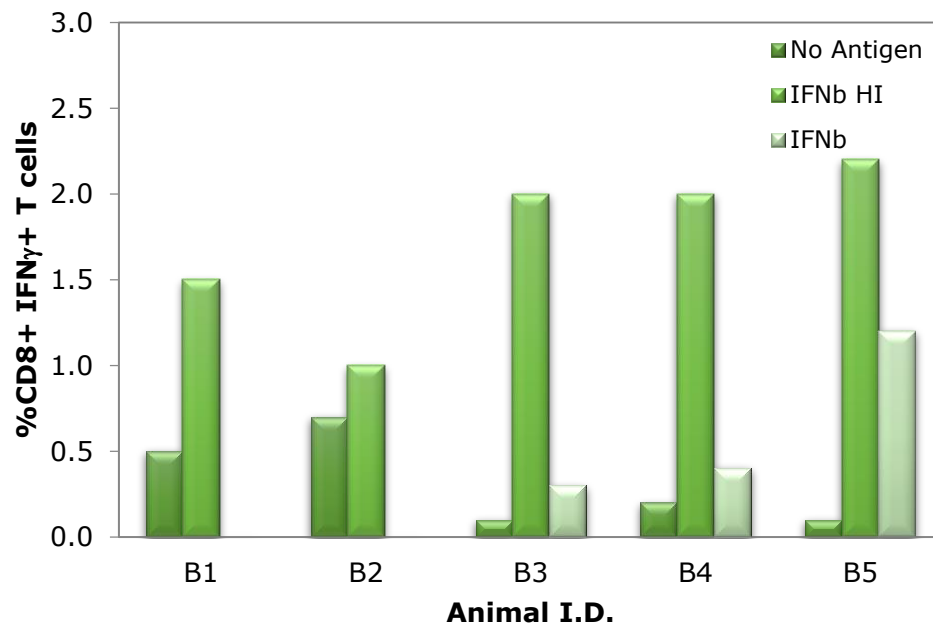
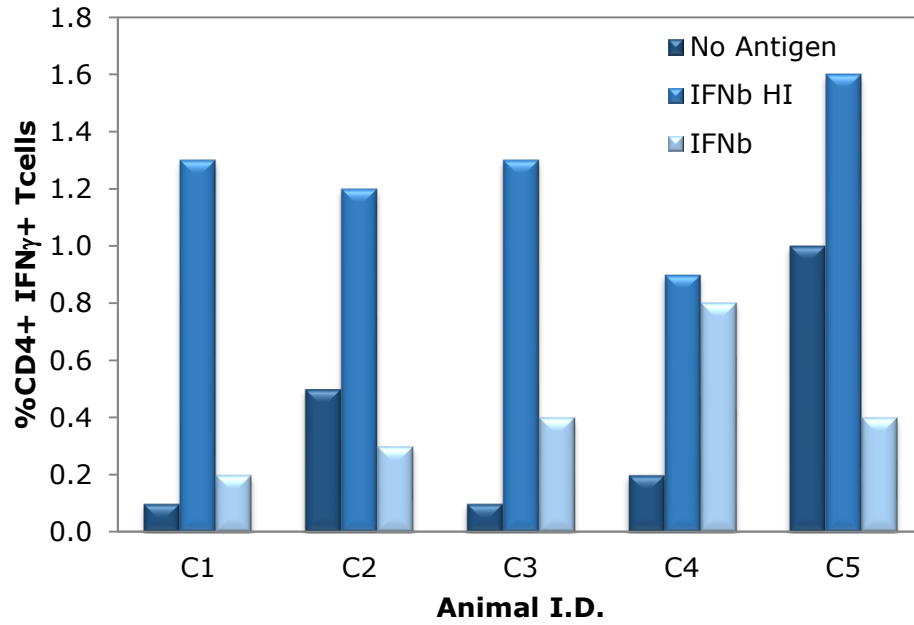


Figure 3.10: Memory T cell responses to human Interferon-beta in ABH Biozzi mice. Biozzi ABH mice were immunized with **70 µg** Betaferon® (n=5) in complete Freund's adjuvant on days 0, 7 and 21; splenocytes were tested in the intracellular cytokine assay on day 38. An IFNβ-specific IFNγ response by memory CD4 (A) and CD8 (B) T cells was observed 4 of the 5 test mice over control responses (no antigen controls).

A)



B)

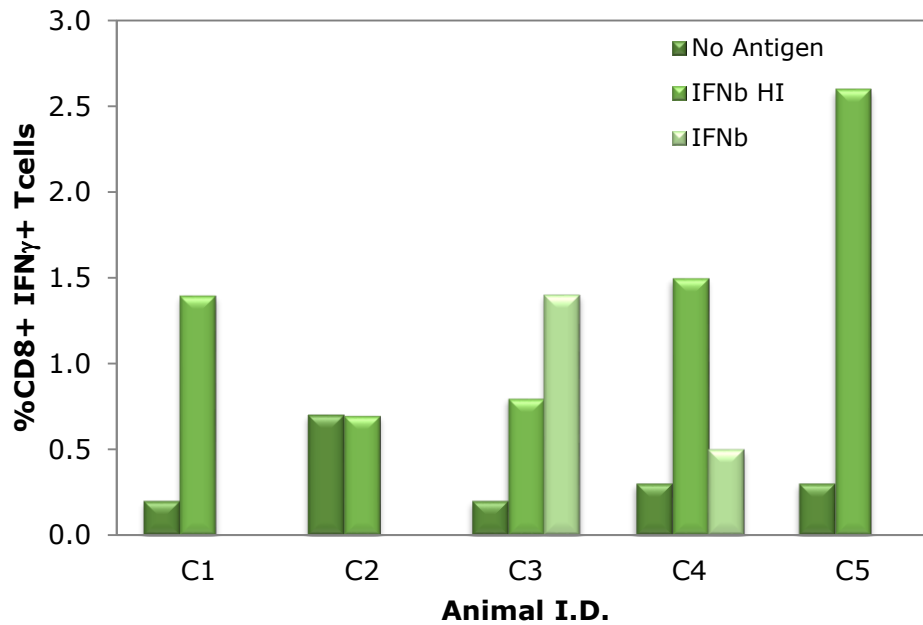
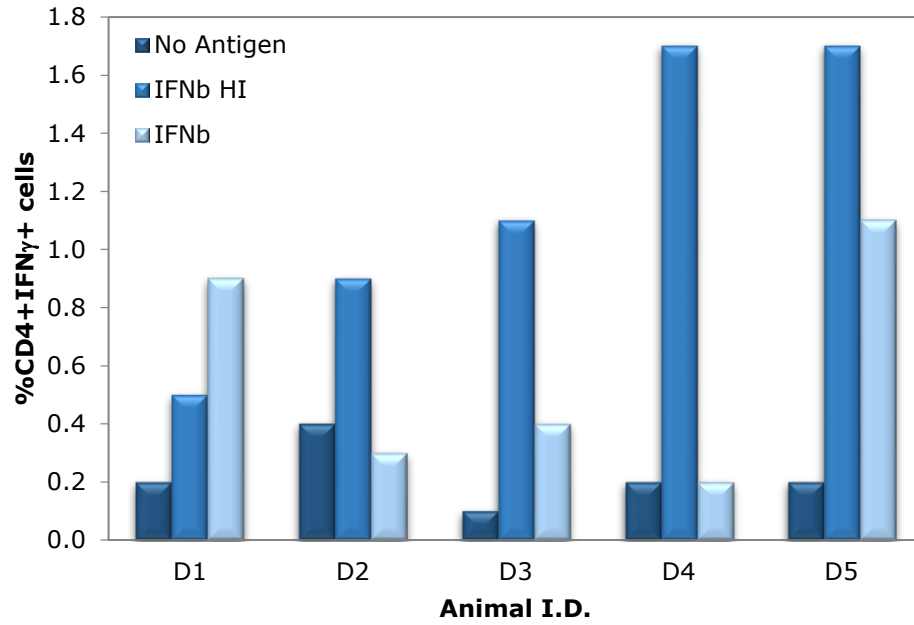


Figure 3.11: Memory T cell responses to human Interferon-beta in ABH Biozzi mice. Biozzi ABH mice were immunized with **50 µg** Betaferon® (n=5) in complete Freund's adjuvant on days 0, 7 and 21; splenocytes were tested in the intracellular cytokine assay on day 38. An IFNβ-specific IFNγ response by memory CD4 (A) and CD8 (B) T cells was observed in 4 of the 5 test mice over control responses (no antigen controls).

A)



B)

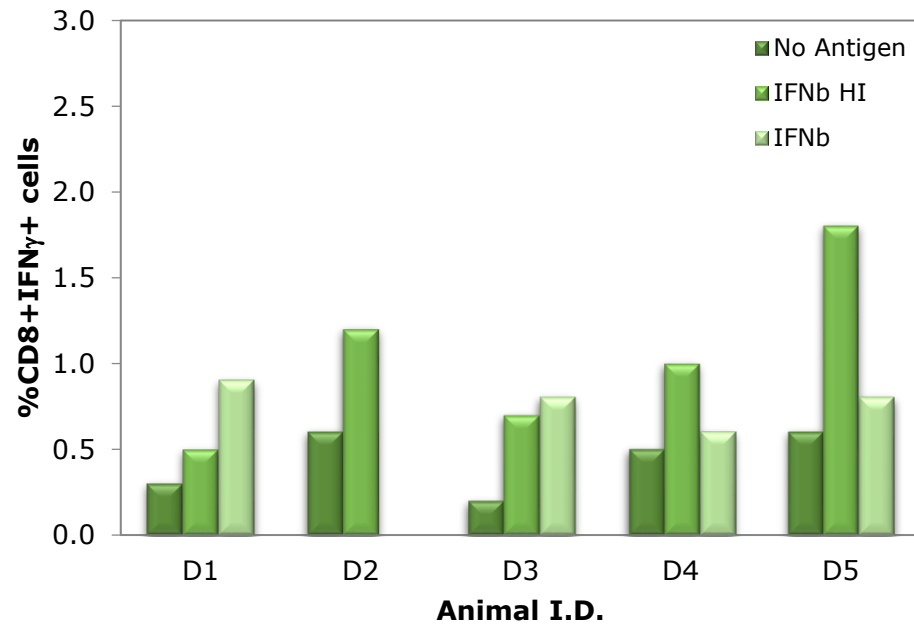


Figure 3.12: Memory T cell responses to human Interferon-beta in ABH Biozzi mice. Biozzi ABH mice were immunized with **20 μ g** Betaferon® (n=5) in complete Freund's adjuvant on days 0, 7 and 21; splenocytes were tested in the intracellular cytokine assay on day 38. A significant IFN β -specific IFN γ response by memory CD4 (A) and CD8 (B) T cells was observed in all test mice over control responses (no antigen controls).

3.2.2 Induction of NABs in BALB/c mice

Mice were immunized with recombinant human IFN β (Avonex®, Betaferon® or Rebif®) in CFA via the sub-cutaneous route (section 2.4); and sera/plasma samples collected (section 2.6) and tested in the neutralizing antibody assay (section 2.8). The 50% point between maximum and minimum log LCPS (luminescent counts per second) was determined and used to calculate the percentage inhibition; titres were also calculated for each sample tested (section 2.8.4). Naïve, unimmunized mice serum was used as a negative control.

BALB/c mice (n=15) were immunized subcutaneously on days 0, 7 and 21 with 5, 10 or 20 μ g of human IFN β -1b (Betaferon®) in CFA per immunization. This immunization schedule was chosen as it is used routinely in our lab for induction of EAE in the mouse model. Serum samples were collected on day 2, 14 and 35 (figure 3.13).

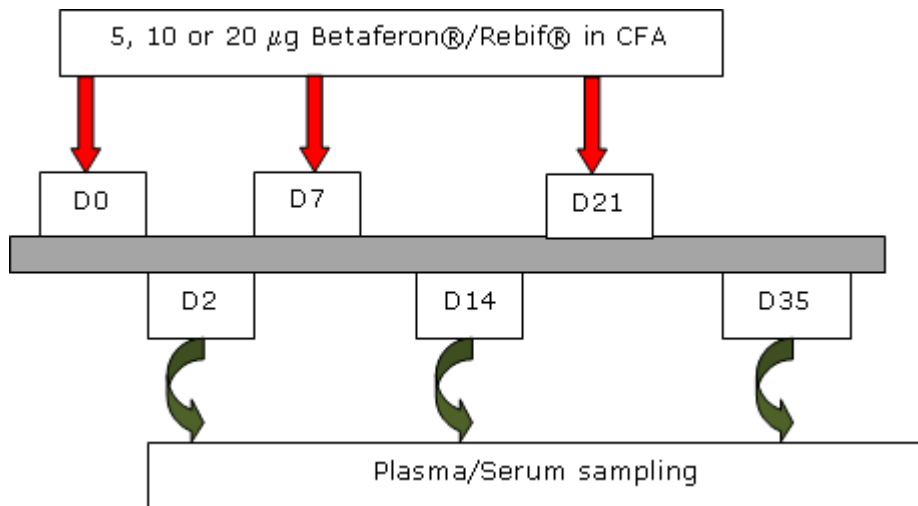


Figure 3.13: Timeline for immunization schedule for the administration of interferon-beta in mice. BALB/c mice were immunized with 5, 10 or 20 μ g Betaferon® (n=15) in complete Freund's adjuvant on days 0, 7 and 21. Plasma/serum samples were taken on Days 2, 14 and 35.

The serum samples were tested in the NAbs assay, no NAbs were observed on days 2 and 14 above the negative control (data not shown). NAbs were observed in 2 mice immunized with 20 µg Betaferon® on Day 35 (table 3.2).

	5 µg		10 µg		20 µg	
	%Inhibition	Titre	%Inhibition	Titre	%Inhibition	Titre
1	0	<20	52.9	22.5	92.7	55.1
2	15.2	<20	26.9	<20	0	<20
3	0	<20	12.9	<20	80.9	43.9
4	0	<20	15.6	<20	7.9	<20
5	0	<20	34.1	<20	44.1	<20

Table 3.3: Neutralizing antibodies to Interferon-beta in BALB/c mice. BALB/c mice (n=15) were immunized with 5, 10 or 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; plasma samples were tested in the NAbs assay on day 35. %inhibition is the percentage of IFNβ that was inhibited by the sample upon incubation. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. NAbs to IFNβ was observed in 2 of the higher dosed test mice.

Terminal splenocytes were tested in the intracellular cytokine assay. No significant IFNβ-specific CD4 T cells responses were observed (data not shown). Figure 3.14 shows the CD8 T cell responses observed. An IFNβ-specific IFNγ response by CD8 T cells was observed in all test mice but not significantly higher than the controls (no antigen).

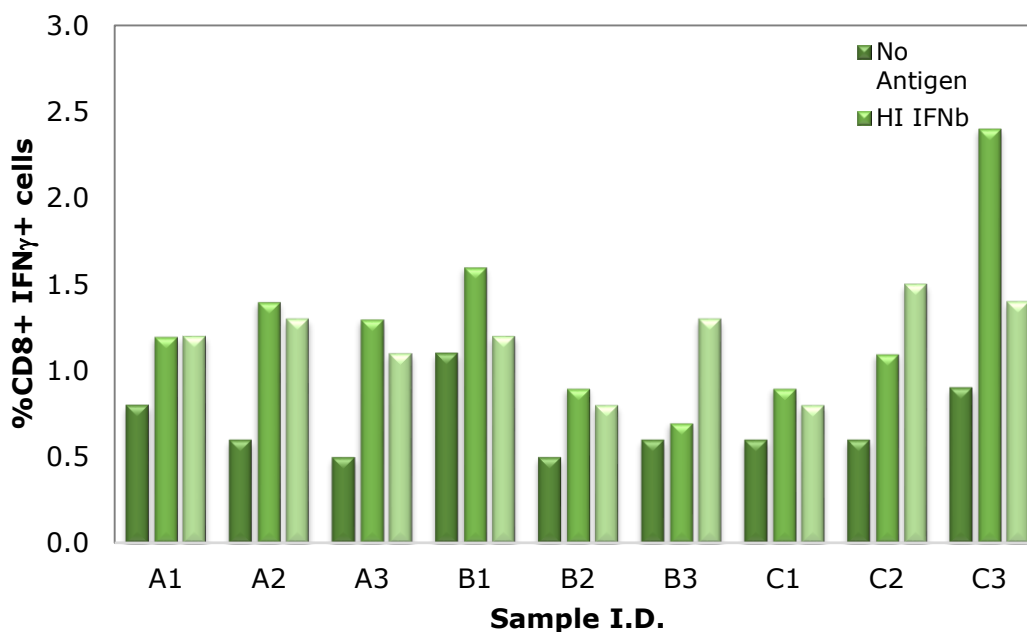


Figure 3.14: CD8 T cell responses to human Interferon-beta in BALB/c mice. BALB/c mice (n=15) were immunized with 5 (A), 10 (B) or 20 (C) μ g Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; splenocytes were tested in the intracellular cytokine assay on day 35. No antigen was used as a control. An IFN β -specific IFN γ response by CD8 T cells was observed in all test mice over control responses (no antigen controls).

Following the results observed in table 3.2, mice were immunized with the higher dose of Betaferon® to see if NAbs could be established in this model. BALB/c mice (n=15) were immunized with 20 μ g Betaferon® and samples taken on day 35 (2 weeks post the 3rd immunization) and tested in the NAbs assay. 13 of the 15 mice immunized were NAbs positive (table 3.3).

	20 µg		20 µg		20 µg	
	%Inhibition	Titre	%Inhibition	Titre	%Inhibition	Titre
1	95.8	>320	88.1	>320	76.7	131.97
2	94.1	>320	94.1	>320	0.6	<20
3	91.8	>320	94.7	>320	79.9	103.1
4	90.2	>320	86.1	>320	48.6	<20
5	95.6	>320	95.4	>320	50.1	23.0

Table 3.4: Neutralizing antibodies to Interferon-beta in BALB/c mice.

BALB/c mice (n=15) were immunized with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; plasma samples were tested in the NAbs assay on day 35. %inhibition is the percentage of IFNβ that was inhibited by the sample upon incubation. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. NAbs to IFNβ was observed in 13 of 15 test samples.

Table 3.3 is a representative result of several experiments testing for NAbs in the BALB/c mice. Similar results were consistently observed in the BALB/c mice following immunization with 20 µg Betaferon® on days 0, 7, and 21. As a result BALB/c mice were immunized using this protocol in subsequent experiments.

To investigate the involvement of CFA in induction of NAbs to rhIFNβ, BALB/c mice (n=8) were immunized on days 0, 7 and 21 with CFA only. Serum samples were taken on day 35 and tested in the NAbs assay. No NAbs were observed in these mice (figure 3.15).

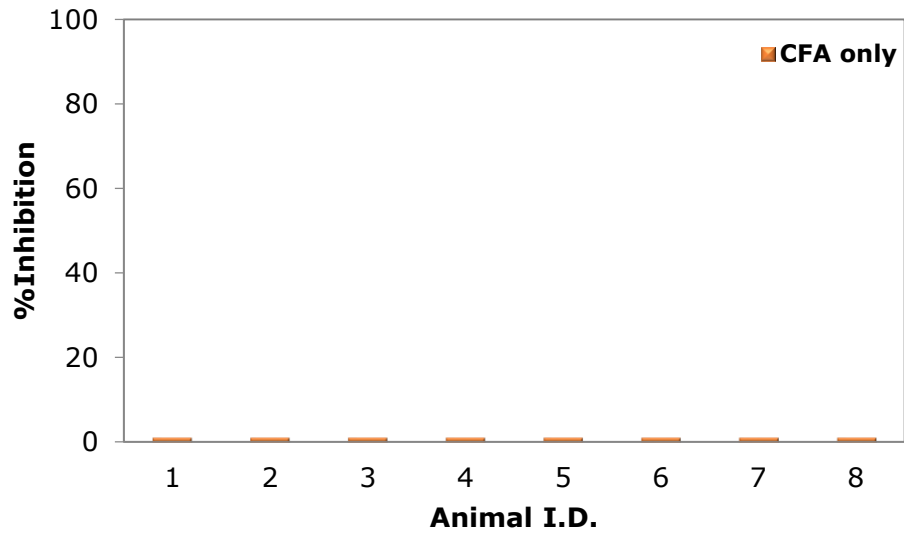


Figure 3.15: Neutralizing antibodies to Interferon-beta in CFA immunized BALB/c mice. BALB/c mice (n=8) were immunized sub-cut with complete Freund's adjuvant on days 0, 7 and 21; plasma samples were tested in the NAbs assay on day 35. %inhibition is the percentage of IFN β that was inhibited by the sample upon incubation. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. No NAbs to IFN β were observed in all mice tested.

3.3 Discussion

This chapter details the establishment of a mouse NAbs model in the context of rhIFN β , and its potential use in investigating strategies for inducing tolerance to rhIFN β in this model (discussed in chapter 5) and indirectly in IFN β -treated MS patients with NAbs. The Biozzi ABH mouse and BALB/c mice were both investigated for the establishment of the NAbs model. The BALB/c was used as it more consistently had NAbs to IFN β in comparison to the ABH mice.

It was expected that administration of rhIFN β in the mouse would result in an immune response as the rhIFN β is analogous to human IFN β and not mouse IFN β . IFN β is anti-proliferative to certain cells but did not show cross-species anti-proliferative effect in a xenograft mouse model (Qin *et al.*, 2001). IFN β is readily cleared from circulation and the mode of administration affects the clearance of IFN β : subcutaneous and intramuscular administration will enhance an immune response to IFN β in comparison with oral, mucosal or intravenous routes which should induce tolerance to the protein on administration. The Biozzi ABH mice and the BALB/c mice were the 2 mouse models chosen as they produce high levels of antibodies in an immune response. Therefore by investigating these 2 mouse models as potential NAbs models, it was hoped that results obtained in the development of NAbs in these mouse strains may be predictive of the responses to IFN β observed in MS patients. The Biozzi ABH mice are used extensively in our lab as an EAE model, and this was the main reason it was chosen as a model for the establishment of NAbs to rhIFN β . The ABH mice produce high levels of antibody in response to an antigen. The ABH mice were initially immunized with Rebif®, and NAbs to rhIFN β were observed in these mice. Results observed also showed that the NAbs were not long lasting and it had previously been shown that the half-life of

IFN β is short (Salmon *et al.*, 1996). Further immunization with Rebif® did not induce NAbs in all the ABH mice immunized. Betaferon® was chosen next as it is generally more immunogenic in humans than the Rebif® or Avonex® (Kagawa *et al.*, 1988). The different concentrations of Betaferon® (high and low) did not appear to consistently induce a detectable level of NAbs in the Biozzi ABH mice. NAbs to rhIFN β has previously been observed in BALB/c mice (Bellomi *et al.*, 2007). In the study, the mice were immunized with Avonex® or Rebif® (old and new formulations) and NAbs was observed on Day 35. They concluded that there were differences in the immunogenicity of different formulations of IFN β available: the Rebif® new formulation was less immunogenic than the current formulation of Rebif® and Avonex® in the BALB/c mouse model. Betaferon® has been shown to induce NAbs more readily in humans than the other formulations of IFN β and was the chosen formulation to be used in the development of NAbs in the BALB/c mouse model. The mice were initially immunized with low concentrations of Betaferon® and NAbs were detected in some of the mice, some of the time. On immunizing with 20 μ g/ml Betaferon®, NAbs were consistently observed in more than 70% of mice by day 35.

A human IFN β transgenic mouse model has been developed (Hermeling *et al.*, 2005). The model expressed the human IFN β gene and immune responses to human IFN β were limited in these mice. The human IFN β transgenic immune-tolerant C57Bl/6 mice were used to study the effect of oxidation on the structure of rhIFN β and its immunogenicity (van Beers *et al.*, 2011). The authors showed that oxidation and aggregation of human IFN β resulted in NAbs formation by the breaking of tolerance. The main difference between this transgenic model and the NAbs model in this chapter is that these transgenic mice are resistant to rhIFN β and would mount a response by breaking of tolerance. In the NAbs model on the other

hand, an immune response is mounted as a result of the rhIFN β being seen as a foreign protein. In MS patients with NAbs, IFN β is seen as foreign and an immune response is mounted to the protein (Hermeling *et al.*, 2005). The study of NAbs in the context of IFN β was chosen rather than MS directly to investigate the effects of tolerization strategies on the outcome of NAbs and indirectly on the outcome of IFN β treatment in MS patients with NAbs to the protein. In MS, autoimmunity is unproven, the auto-antigens are not well defined and if the tolerization strategy goes wrong, it could exacerbate the disease (Vergelli *et al.*, 1996); while in NAbs positive patients on IFN β treatment, autoimmunity is proven, the auto-antigen and time course for developing antibodies is known and patients that already have a NAbs response are not physically unwell. The loss of endogenous IFN β activity as a result of NAbs does not cause disease but has been shown to reduce the availability of IFN β and hence its bioactivity (Francis *et al.*, 2005). The NAbs model was used to study tolerization strategies (discussed in chapter 5) which was hoped could be extrapolated for use in IFN β -treated MS patients with NAbs to improve and hopefully prolong MS treatment with IFN β .

Initial exposure to a foreign protein, in this study human IFN β leads to T cells being primed followed by a rapid expansion of these effector cells. A proportion of these effector cells differentiate into a memory population that are antigen experienced and can be long lasting. These memory cells respond rapidly to a secondary exposure to the same antigen. In this study, memory CD4 and CD8 T cells responded to IFN β as was observed by their proliferative responses and IFN γ production. Prime-boost protocols in mice have been shown to increase the memory T cell pool, especially the CD4 T cells and this has been shown to transiently accelerate the development of NAbs. Though there was a T cell response to IFN β in the ABH mice, detectable NAbs were not observed in these immunized mice.

In the ABH model, NAbS were observed in initial studies but we failed to observe detectable NAbS consistently in this model. On the other hand more consistent NAbS positivity was observed in the BALB/c mouse model. NAbS were consistently observed (70-100% positive) on Day 35 in mice immunized with 20 µg/ml Betaferon® on days 0, 7 and 21.

In conclusion the BALB/c mouse model was used in the development of the NAbS model. This model will help investigate the induction of tolerance to IFNβ and the reduction/elimination of NAbS in the model, it is hoped that the strategy will be useful in MS patients that have developed NAbS following IFNβ treatment.

Chapter 4

Development of depletion strategy

4.1 Introduction

There are several methods that can be used for depleting cells of the immune system. In this project, mitoxantrone was used to deplete different immune cell subsets; furthermore, I used anti-CD4 depleting antibody and anti-CD20 depleting antibody, which deplete CD4 T cells and B cells respectively.

The depletion of the immune cells by mitoxantrone, anti-CD4 antibody and anti-CD20 antibody triggers a reconstitution program that leads to renewal of the T cell and B cell repertoire. Within this window of reconstitution, it is hoped that the immune system can be manipulated or “re-educated” towards a tolerogenic response to a particular antigen. In this chapter the antigen of interest is human interferon beta.

4.1.1 Anti-CD4 antibody

CD4 is a marker found on a subset of T cells in the immune system. CD4 T cells are involved in cellular and humoral immunity through cytokine secretion and also via cell-cell interaction. EAE and MS are thought to be T cell mediated diseases and CD4 T cells have been extensively studied in these diseases (Zamvil *et al.*, 1990). CD4 depletion has been used to investigate the involvement of CD4 T cells in the development, pathogenesis and progression of EAE. Administration of YTS177 (a non-depleting CD4 monoclonal antibody) results in the down regulation of CD4 which is required for T cell activation; and administration of YTS191.1 (CD4-specific mAb) led to a rapid reduction of CD4 T cells in the peripheral blood and lymphoid tissue (Cobbold *et al.*, 1984). CD4-specific monoclonal antibody (mAb) suppresses T

cell dependent responses and has been shown to prevent the development of EAE (Cobbold *et al.*, 1984). Selective elimination of CD4 T cells have been trialed in MS patients using a chimeric mAb cM-T412 (Rep *et al.*, 1997), but the animal results could not be directly translated to MS. Treatment resulted in depletion of CD4 T cells and these were long-lasting in these patients, however beneficial clinical effects measured by the number of active lesions by MRI were lacking (van Oosten *et al.*, 1997). In this project, CD4 T cells were depleted in blood and in spleen using anti-CD4 depleting antibody.

4.1.2 Anti-CD20 antibody

B cells are an active part of the immune system. B cells develop in the bone marrow and then migrate to periphery and to secondary lymphoid organs (Browning, 2006). On encountering antigen, B cells proliferate and differentiate into antibody secreting plasma cells which can produce antibodies for weeks or months. Memory B cells are formed with the progression of an immune response and these provide a rapid antibody response upon a second encounter with the same antigen (Blink *et al.*, 2005).

MS is considered to be primarily a T cell mediated disease, emerging evidence have indicated that B cells may play an important role in pathogenesis of disease both as antibody-secreting plasma cells and as antigen-presenting cells (APC) for the activation of T cells (Weber *et al.*, 2010). Furthermore, clonally expanded B cells, plasma cells and antibodies to a number of MS antigens have been found in the central nervous system (CNS). Oligoclonal bands (OCBs) are an important diagnostic marker for MS. OCB are immunoglobulins and their presence in the cerebrospinal fluid (CSF) indicates inflammation. However, the nature of OCBs remains a mystery and their antigenic targets have yet to be established (Correale *et al.*, 2002).

CD20 is a surface marker expressed by B cells from the pre-B cell phase to the mature B cell phase and the marker is lost during differentiation into plasma cells (Tedder *et al.*, 1994). Rituximab® depletes a subpopulation of B cells (pre-B and mature B cells) without affecting antibody production and secretion (Petereit *et al.*, 2008). Rituximab is a humanized mouse monoclonal antibody against the human CD20 surface molecule expressed by B cells. Rituximab® has been shown to reduce relapse rate by about 50% in relapsing-remitting MS (Bar-Or *et al.*, 2008) and is an exciting new treatment for MS. Interestingly, the positive effect was already seen after 12 weeks, which may highlight that Rituximab may act via direct B cell lysis or inflammatory mechanisms rather than by reducing the level of pathogenic autoantibodies. In mice, it has been shown that Rituximab depletes B cells in the peripheral blood, secondary lymphoid organs and in the CNS. EAE severity was reduced in a transgenic EAE model treated with Rituximab prior to immunization or at the onset of the clinical signs (Monson *et al.*, 2011).

Mature and memory B cells were successfully depleted using anti-CD20 antibody and this abrogated adaptive humoral immunity in a mouse model (DiLillo *et al.*, 2008). To investigate antibody responses to IFN β in mice, B cells were depleted using anti-CD20 antibody.

4.1.3 Mitoxantrone

Mitoxantrone is an anthracenedione which acts by intercalating into the DNA molecule, it also interferes with RNA, and uncoiling and repair of damaged DNA by inhibiting topoisomerase II enzyme (Fox, 2006). Mitoxantrone acts in a dose-dependent manner and is a potent immunosuppressive agent which inhibits *in vivo* induced proliferative response (Baker *et al.*, 1992).

Mitoxantrone leads to broad immunosuppression and causes a reduction in CD4 and CD8 T cells, macrophages and B cells in the spleen (Piao *et al.*, 2007), and has been

shown to significantly reduce the severity of acute phase EAE and also can prevent the onset of EAE if used pre-induction in a dose-dependent manner (Baker *et al.*, 1992). Clinical trials data also suggest efficacy of mitoxantrone in reducing relapses and slowing disease progression in relapsing-remitting and secondary progressive MS (Debouverie *et al.*, 2007). A nonspecific cytotoxicity effect on lymphocytes is believed to be a major mechanism responsible for the clinical effects of mitoxantrone in MS patients (Neuhaus *et al.*, 2005).

4.2 Results

4.2.1 Anti-CD4 depletion in BALB/c

Depletion of CD4 T cells was investigated in the blood and spleen at various time points following a single i.p. administration of 200 µg/mouse anti-CD4 antibody in naïve BALB/c mice (section 2.3). The time points were days 3, 7, 16 and 21 post i.p. administration. The volume for administration and the time points were chosen from results obtained by Pryce et al (Pryce *et al.*, 2005). Blood and splenocytes were stained for T cell markers (CD4 and CD8), B cell markers (B220) and macrophage markers (F4/80) as detailed in section 2.7. CD4 T cells were gated (figure 4.1) in naïve samples as well as the time points investigated. Figure 4.1 shows representative plots observed. CD4 and CD8 T cells, B cells and macrophage cell percentages in blood (figure 4.2) and in spleen (figure 4.3) were calculated and naïve unimmunized mice were used as controls. A significant reduction in CD4 T cell proportions were observed in blood and in the spleen on all the days sampled. The proportion of CD4 T cells appeared to be rising on day 21 but had not reached the level observed in the naïve mice. A reduction in B cells in the blood and a significant reduction in macrophages in the spleen were also observed.

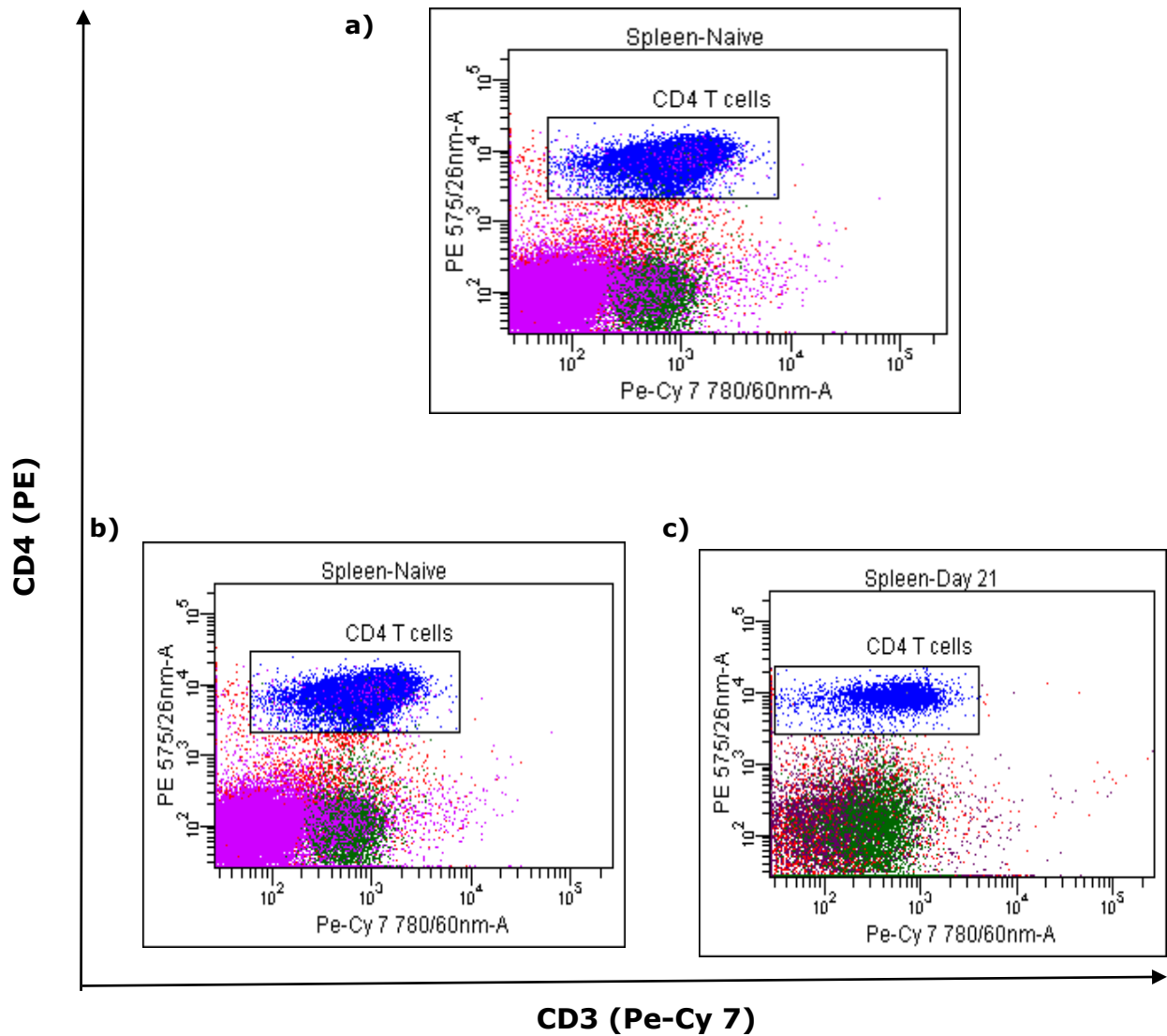


Figure 4.1: CD4 T cell phenotyping in BALB/c mice following administration of anti-CD4 antibody. Flow cytometry analysis of CD4 T cell population in naïve BALB/c mice post administration of anti-CD4 antibody. Splenocytes were stained for CD4 T cell markers; CD4 T cells were gated in the lymphoid population to determine proportions in naïve (a), on day 3 (b) and on day 21 (c) post administration of anti-CD4 antibody.

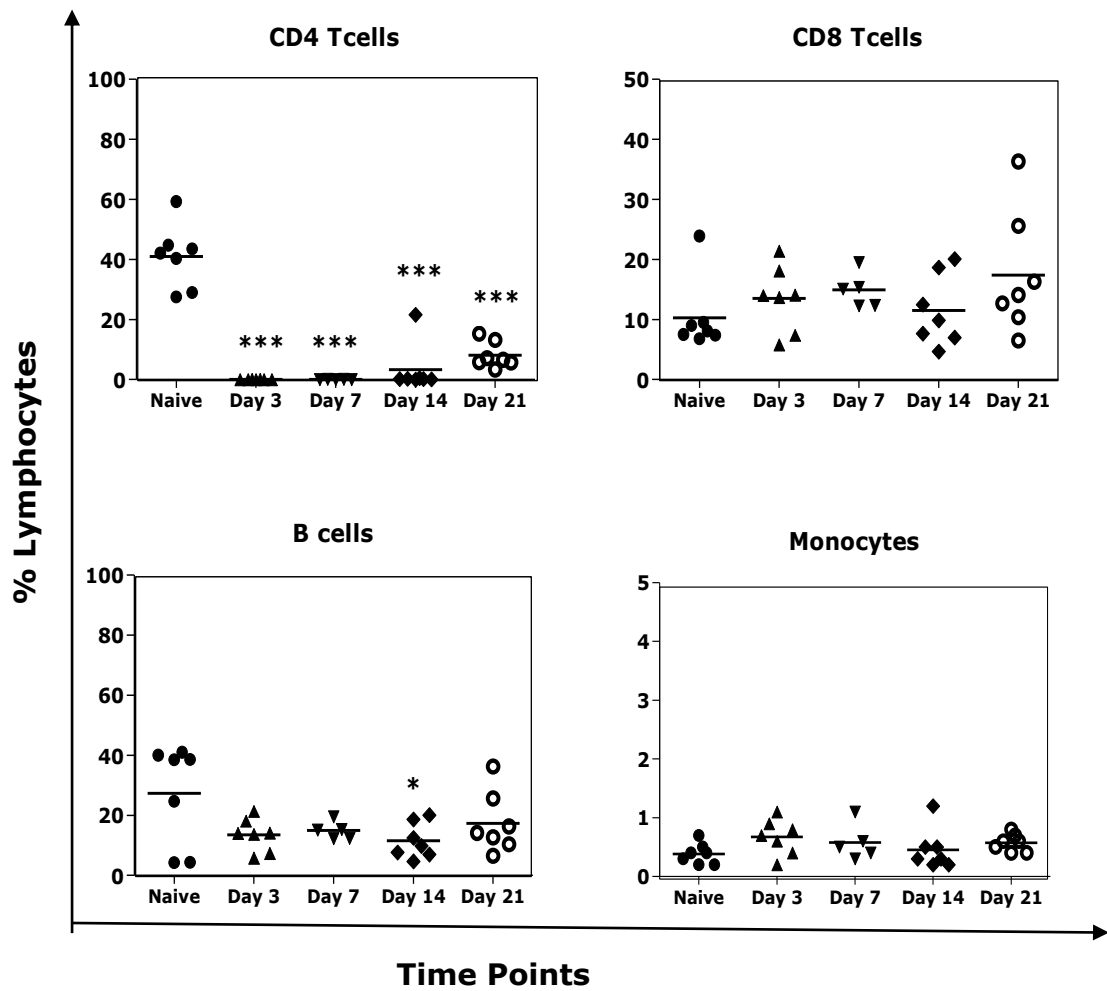


Figure 4.2: Cell proportions in blood following administration of anti-CD4 antibody in naïve BALB/c mice. Naïve BALB/c mice were given i.p. 200 μ g/mouse anti-CD4 antibody, blood samples harvested and proportions of B cell, CD4 and CD8 T cells, and monocytes calculated on days 3, 7, 14 and 21. *P<0.05 compared to controls, **P<0.005 compared to controls and ***P<0.0005 compared to controls.

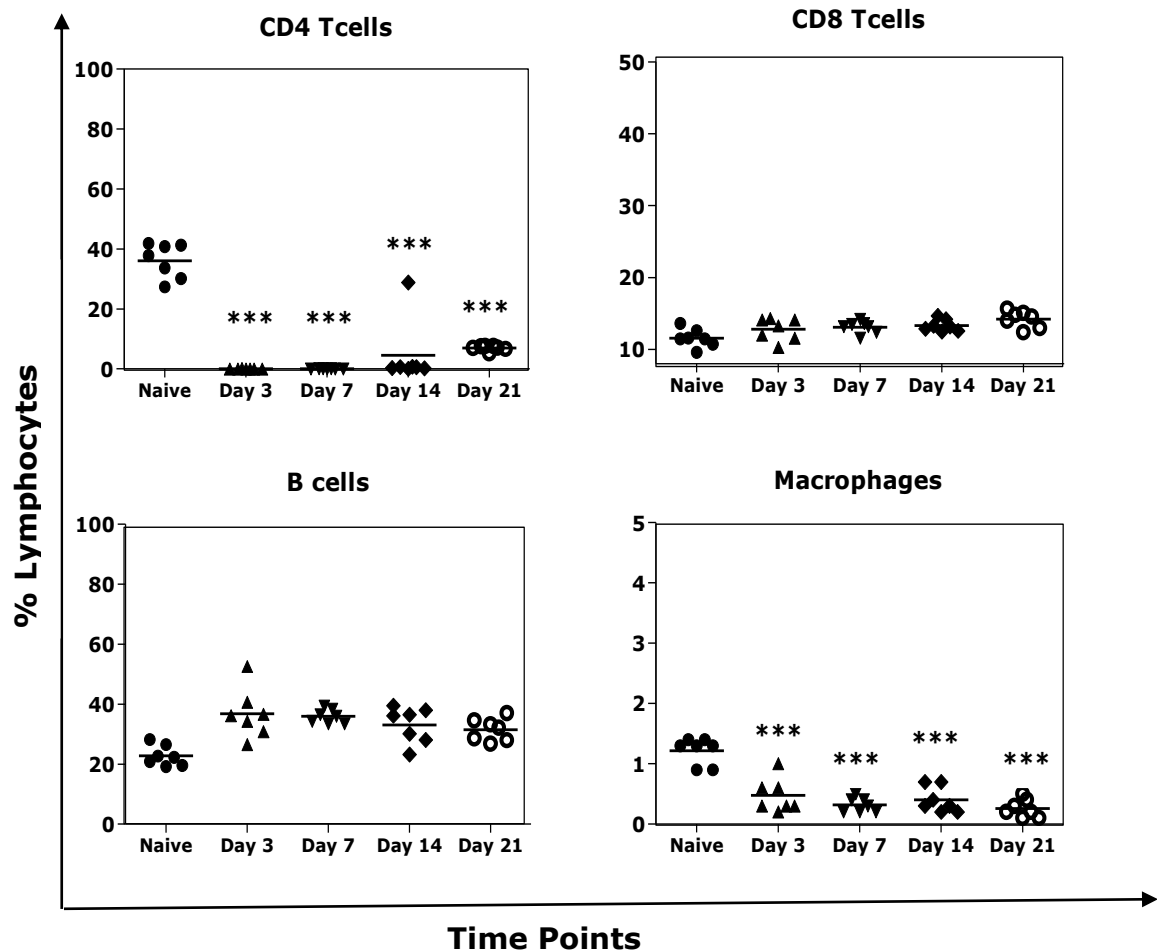


Figure 4.3: Cell proportions in the spleen following administration of anti-CD4 antibody in naïve BALB/c mice. Naïve BALB/c mice were given i.p. 200 µg/mouse anti-CD4 antibody, spleens harvested and proportions of B cells, CD4 and CD8 T cells, and macrophages calculated on days 3, 7, 14 and 21. * $P < 0.05$ compared to controls, ** $P < 0.005$ compared to controls and *** $P < 0.0005$ compared to controls.

4.2.2 Anti-CD20 antibody in BALB/c mice

Depletion of B cells was investigated in the blood and spleen at various time points following a single i.v. administration of 250 µg/mouse anti-CD20 antibody in naïve mice (section 2.3). The time points were days 7, 14 and 21 post administration of the antibody. Anti-CD20 antibody was a gift from Dr Dunn and the volume and the time points were a recommendation by Dr Dunn. Blood and splenocytes were

stained for T cell markers (CD4 and CD8), B cell markers (B220) and monocyte markers (F4/80) detailed in section 2.7. CD4 and CD8 T cells, B cells and macrophage cell percentages in blood (figure 4.4) and spleen (figure 4.5) were calculated and naïve unimmunized mice were used as controls. There was a significant reduction in B cell proportions in blood and spleen on all the days sampled, the reduction was more pronounced in the spleen than in the blood. In the spleen, B cell proportions remained low even at day 21. A reduction in CD4 and CD8 T cells in the blood were also observed in these mice.

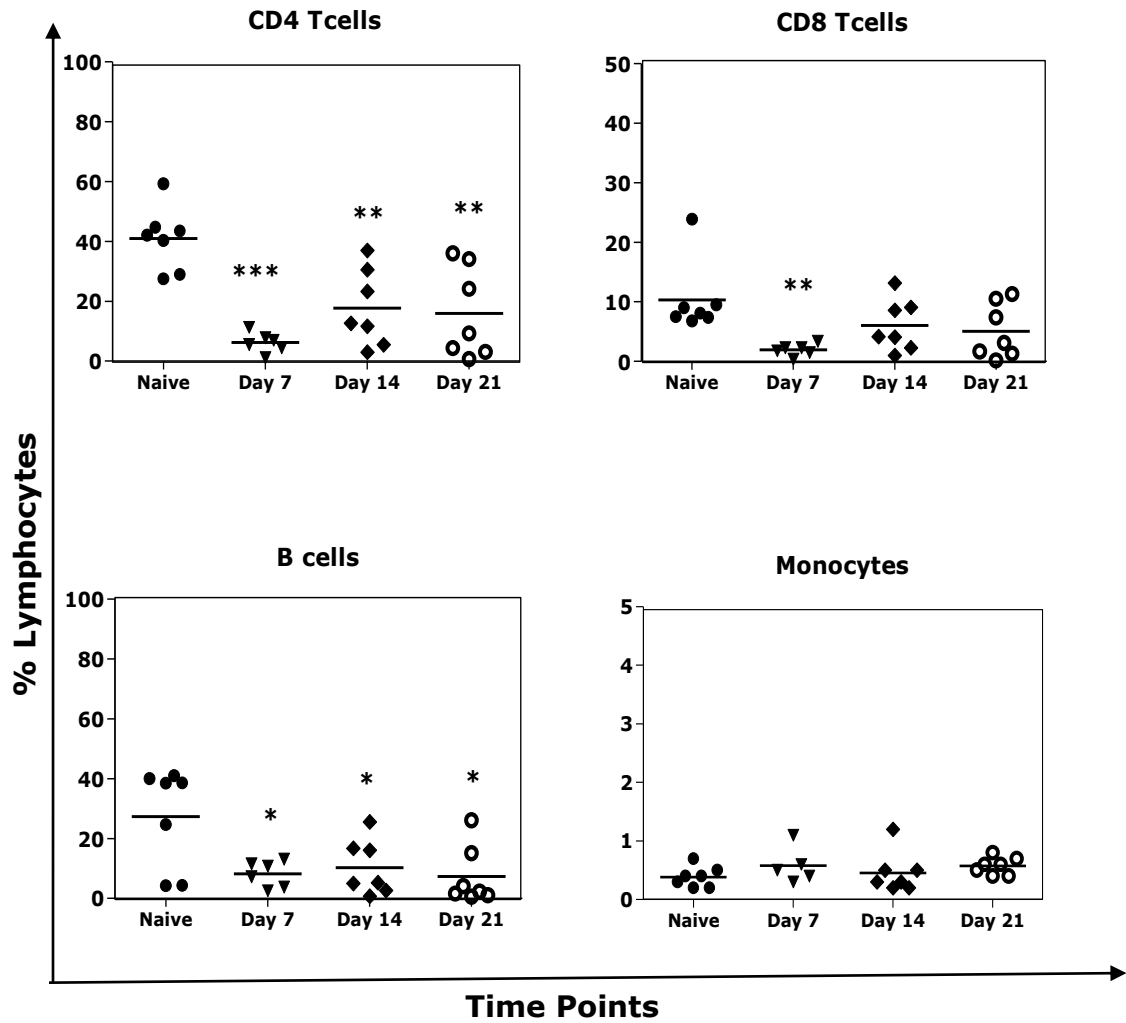


Figure 4.4: Cell proportions in blood following administration of anti-CD20 antibody in naïve BALB/c mice. Naïve BALB/c mice were given i.v. 250 µg/mouse anti-CD20 antibody, blood harvested and proportions of B cell, CD4 and CD8 T cells, and macrophages calculated on days 7, 14 and 21. * $P < 0.05$ compared to controls, ** $P < 0.005$ compared to controls and *** $P < 0.0005$ compared to controls.

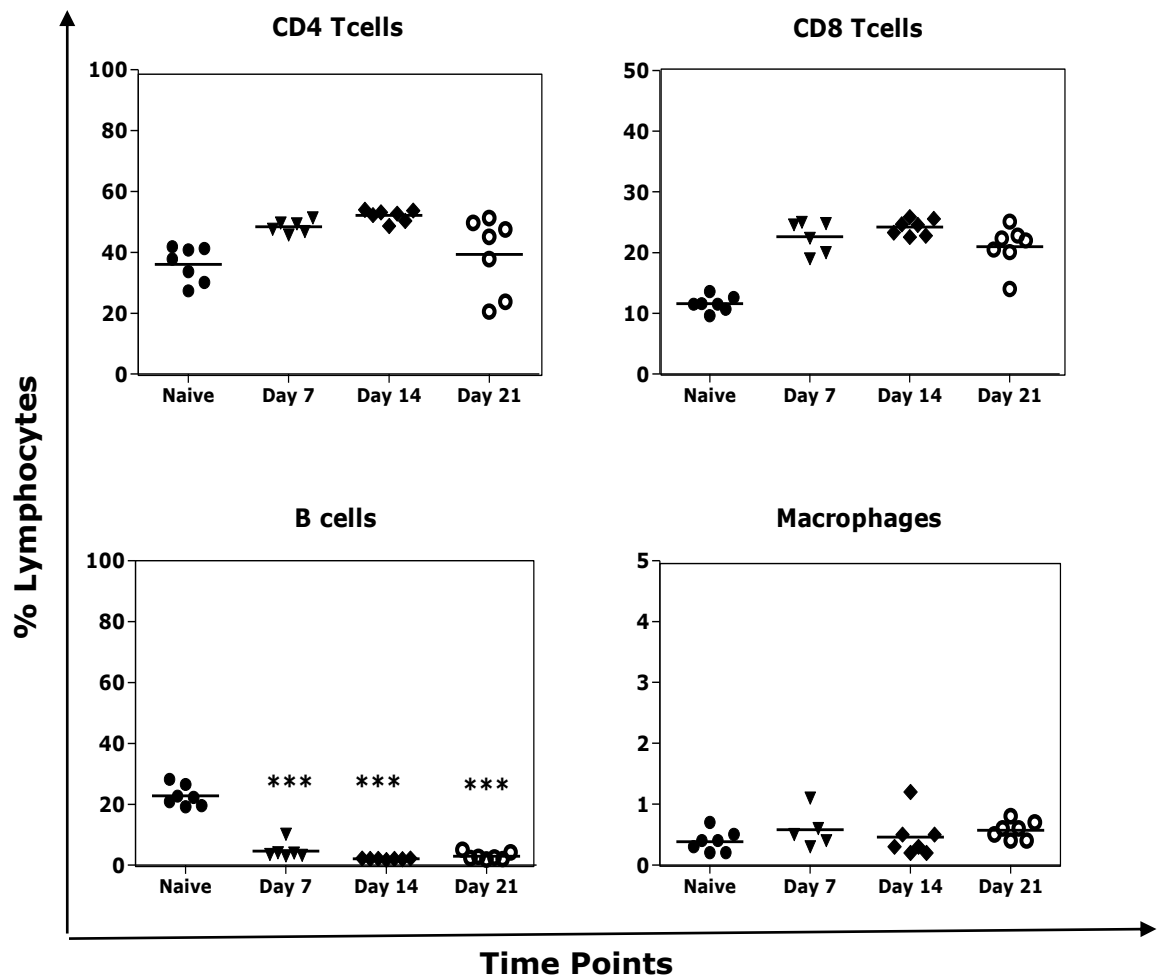


Figure 4.5: Cell proportions in spleen following administration of anti-CD20 antibody in naïve BALB/c mice. Naïve BALB/c mice were given i.v. 250 µg/mouse anti-CD20 antibody, spleen harvested and proportions of B cell, CD4 and CD8 T cells, and macrophages calculated on days 7, 14 and 21. * $P < 0.05$ compared to controls, ** $P < 0.005$ compared to controls and *** $P < 0.0005$ compared to controls.

4.2.3 Mitoxantrone in BALB/c mice

Depletion of cells was investigated in the blood and spleen at various time points following a single i.p. administration of 2.5 mg/kg mitoxantrone in naive mice (section 2.3). The time points were days 3, 7, 14 and 21 post immunization. The volume of mitoxantrone used was extrapolated from the volume used in treating MS patients. Blood and splenocytes were stained for T cell markers (CD4 and CD8), B

cell markers (B220) and monocyte markers (F4/80) detailed in section 2.7. CD4 and CD8 T cells, B cells and macrophage cell percentages in blood and spleen were calculated and naïve unimmunized mice were used as controls. There was a reduction in CD4 T cells and B cells in the blood but did not reach a significant level (figure 4.6). There was significant reduction in CD4 and CD8 T cell proportions in the spleen on days 14 and 21 after administration of mitoxantrone (figure 4.7). Macrophages and monocytes were significantly reduced in the spleen and blood.

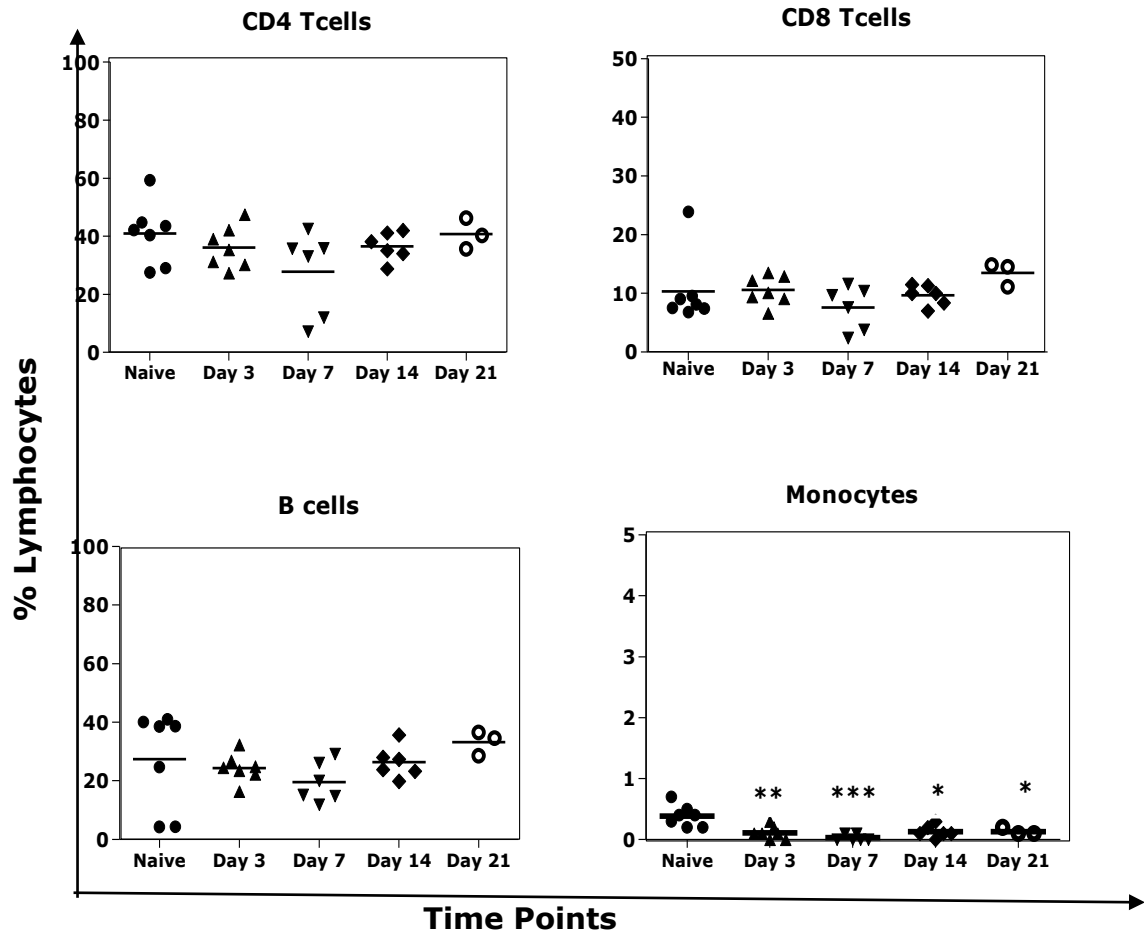


Figure 4.6: Cell proportions in the blood following administration of mitoxantrone in naïve BALB/c mice. Naïve BALB/c mice were given i.p. 2.5 mg/kg mitoxantrone, blood samples harvested and proportions of B cell, CD4 and CD8 T cells, and monocytes calculated on days 3, 7, 14 and 21. * $P < 0.05$ compared to controls, ** $P < 0.005$ compared to controls and *** $P < 0.0005$ compared to controls.

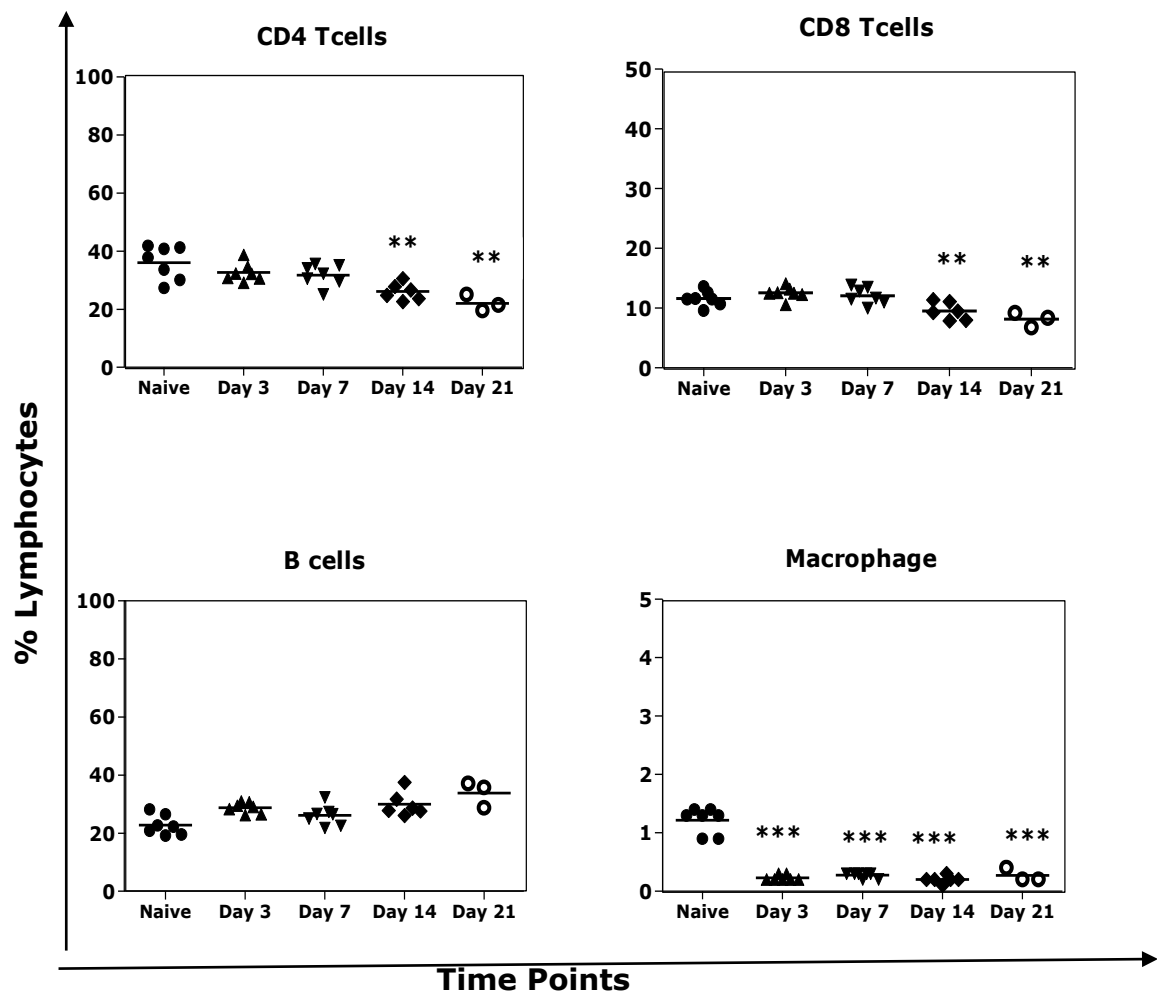


Figure 4.7: Cell proportions in the spleen following administration of mitoxantrone in naïve BALB/c mice. Naïve BALB/c mice were given i.p. 2.5 mg/kg mitoxantrone, spleens harvested and proportions of B cell, CD4 and CD8 T cells, and macrophages calculated on days 3, 7, 14 and 21. * $P < 0.05$ compared to controls, ** $P < 0.005$ compared to controls and *** $P < 0.0005$ compared to controls.

4.3 Discussion

This chapter details the depletion of different cell subsets of the immune system using mitoxantrone or monoclonal antibodies targeting specific cell populations. Mitoxantrone, anti-CD4 and anti-CD20 antibodies were administered in naïve BALB/c mice to investigate cell subset depletion. Anti-CD4 antibodies significantly reduced CD4 T cells in spleen and blood; and anti-CD20 antibody significantly reduced B cells in spleen and blood. Mitoxantrone significantly reduced CD4 and CD8 T cells in spleen and reduced CD4 T cells and B cells in the blood. It is thought that depletion of immune cell populations leads to a reconstitution of naïve cell pools. It is hoped that during this reconstitution period, the immune system can be reprogrammed.

Depleting anti-CD4 antibodies have been trialed in MS and have shown that CD4 depletion is long-lasting, however this did not appear to have a beneficial clinical outcome on the disease (van Oosten *et al.*, 1997). In the EAE setting, CD4 T cell depletion has been shown to prevent the development of EAE (O'Neill *et al.*, 1993) and it was shown that CD4 T cell depletion was able to intercept the transition from acute-to-chronic EAE when applied to B-cell-deficient animals that just reached the peak of disease severity (Hoehlig *et al.*, 2012). Following CD4 T cell depletion, reconstitution of these cells was observed in humans (Mackall *et al.*, 1995) and in mice (Rice *et al.*, 1995). In my project, a significant reduction in CD4 T cell proportions were observed in blood and in spleen on all the days sampled following anti-CD4 antibody administration and this was long lasting (21 days). This was also observed in MS patients where, CD4 T cells were depleted long term but this did not appear to affect the outcome of the disease in these patients (van Oosten *et al.*, 1997). The proportion of CD4 T cells appeared to be rising on day 21 but had not

reached the level observed in the naïve mice. Interestingly, a reduction in B cells in the blood was also observed.

Anti-CD20 antibodies have been used in the treatment of MS and EAE. In MS, Rituximab, humanized mouse monoclonal antibody against the human CD20 surface molecule has been shown to reduce relapse rates in RR-MS (Bar-Or *et al.*, 2008). Rituximab has also been shown to affect the outcome of EAE in mice, depending on the time of administration and the animal model used. In this project, B cells were significantly reduced in naïve BALB/c over time in blood and spleen and the reduction was more striking in the spleen up to 21 days post administration. Effective B cell depletion were observed following anti-CD20 administration (Chapter 5 of this thesis) and in C57BL/6 up to day 57 (DiLillo *et al.*, 2008). B cells are depleted in the blood, spleen, lymph nodes and gut-associated lymphoid organs by day 7 (Hamaguchi *et al.*, 2005). Anti-CD20 antibody may deplete memory B cells some of which are precursors of antibody-secreting plasma cells, thereby reducing the sources of new antibody-secreting plasma cells specific for particular antigens (in this project the antigen of interest is human recombinant interferon-beta). The long-lasting depletion of B cells as well as depletion of mature B cells will be relevant in the tolerization strategy discussed in chapter 4 of this thesis. Interestingly, we also saw an effect in the proportion of CD4 and CD8 T cells in the blood post-treatment, which raises interesting questions and should be studied in more detail.

Mitoxantrone has been shown to have a cytotoxic effect in lymphocytes. Mitoxantrone depletes several subsets of lymphocytes including CD4 and CD8 T cells, B cells and NK cells and then a reconstitution of these populations was observed (Piao *et al.*, 2007). Mitoxantrone has a dose-dependent effect on the treatment of EAE, it was shown to significantly reduce the severity of acute EAE and

prevent the onset of EAE if administered pre-induction (Baker *et al.*, 1992). Clinical trials data also suggest efficacy of mitoxantrone in reducing relapses and slowing disease progression in relapsing-remitting and secondary progressive MS (Debouverie *et al.*, 2007). A nonspecific cytotoxicity effect on lymphocytes is believed to be a major mechanism responsible for the clinical effects of mitoxantrone in MS patients (Neuhaus *et al.*, 2005). There was a minor reduction in CD4 T cells and B cells in the blood but this did not reach a significant level. There was significant reduction in CD4 and CD8 T cell proportions in the spleen on days 14 and 21 after administration of mitoxantrone. Mitoxantrone was not well tolerated by the BALB/c mice, and this could be due to the cytotoxic cardiac effect that has been described in humans. Once mitoxantrone was administered, the mice were closely monitored.

With the successful depletion of various immune cell populations in BALB/c mice, it is hoped that the NABs model (discussed in chapter 3) can be used to study strategies for inducing tolerance to human IFN β in combination with depletion strategies. Neutralizing antibodies to rhIFN β will be induced in the BALB/c mice and then the immune cells will be depleted and during the start of the recovery period rhIFN β will be re-introduced via the intravenous route to induce tolerance to the antigen. It is hoped that the regeneration and reprogramming of the immune system will lead to a regeneration of the natural regulatory events that curb autoimmune cells in the MS setting. Once the mechanism of a reconstituting immune system is better understood, we may be able to intervene and re-educate the immune system, which may pose a treatment strategy for autoimmune diseases like MS (Muraro *et al.*, 2006).

Chapter 5

Development of Tolerization Strategy

5.1 Introduction

Immune tolerance can be defined as the ability of the immune system to distinguish self from non-self and harmful from harmless molecules. Tolerance is also the inability to mount an immune response to a subsequent challenge with a previously immunogenic antigen. The route of antigen introduction is critical in determining the outcome of an immune response. Intravenous and mucosal administration of antigen for example, favours the induction of tolerance to the antigen (Verbeek *et al.*, 2007).

In this project, neutralizing antibodies (NAbs) to recombinant human interferon beta (rhIFN β) was used as a model human autoimmune disease to test antigen-specific immune tolerance strategies (development of the NAbs model discussed in chapter 3). Depletion of the immune cells by mitoxantrone, anti-CD4 antibody and anti-CD20 antibody triggers a reconstitution program that leads to renewal of the T cell and B cell repertoire (discussed in chapter 4). Within this window of reconstitution in the established NAbs model, it was hoped that the immune system can be manipulated by re-introducing rhIFN β via the intravenous route leading to tolerance to the protein.

A breakdown of immune tolerance is thought to be an underlying factor in human diseases such as autoimmune diseases. Multiple sclerosis (MS) is a chronic neurodegenerative autoimmune disease and the immune system is thought to be a key player in the progression of the disease (Bruck, 2005). To date there are no definitive treatments which cure MS, but there are several therapies which generally

act as immunomodulators or immunosuppressive agents. Treating MS patients by immunosuppression over years may be problematic as different subsets of lymphocytes are involved in normal immune surveillance; removal of one or more of these subsets by selective depletion may be useful in studying and identifying the mechanisms involved in tolerance induction. However, opportunistic infection and tumouregensis are some of the clinical consequences that may arise as a result of long-term immunosuppression. The “ideal” method in treating autoimmune diseases, such as MS, would be an antigen-specific tolerance therapy to silence the pathogenic autoimmunity without generalised immunosuppression. In IFN β treated MS patients with NAbs, it is hoped that induction of tolerance by elimination or reduction of NAbs will improve the efficacy and longevity of IFN β treatment in these patients.

5.1.1 Central tolerance

Central tolerance is the process by which lymphocytes reactive to self-antigen are largely eliminated during development. The main method is by deletion through negative selection. In the case of T cells, central tolerance occurs when the bulk of self-reactive T cells are deleted during thymic development. T cell development occurs in the thymus, where rearrangement of the T cell receptor (TCR) genes occurs leading to the diversity of T cell population. During intrathymic development of T cells, self-antigen loaded in MHC molecules are present and T cells with TCR that bind to these self-antigen undergo one of 2 selections. T cells with low to medium affinity for self-antigen receive survival signals that ligate the TCR and leads to survival of these T cells termed positive selection (Klein *et al.*, 2009). The other selection process termed negative selection leads to apoptosis of T cells with TCR with strong affinity for self-antigen, strong enough to have the potential of being autoimmune disease mediators. This negative selection process is aided by the

transcription factor Aire. Aire is an autoimmune regulator gene and facilitates the thymic expression of tissue specific antigens (Anderson *et al.*, 2011). The ideal outcome of this selection process would be a T cell repertoire with TCRs that may bind to self-MHC molecules but are unresponsive to self-antigen (Griesemer *et al.*, 2010).

B cell development occurs in the bone marrow. Primary development of B cells is defined by the re-arrangement and expression of immunoglobulin (Ig) genes, these are immature B cells which then undergo selection for self-tolerance. Self-reactive B cells are deleted in the bone marrow by clonal deletion (Wardemann *et al.*, 2003). The surviving B cells leave the bone marrow for the periphery; these B cells are immature and have IgM expressed on their cell surface. B cells that survive negative selection differentiate to become mature B cells (naïve cells) that express IgD, IgM and other proteins such as B220 in mice. B220 is expressed in all stages of B cell development in the mouse. The final stages of development of B cells take place in the spleen (Loder *et al.*, 1999); B cells undergo antigen-dependent differentiation into antibody-secreting and/or memory B cells. Developing B cells that bind weakly to self-antigen survive through positive selection (Levine *et al.*, 2000) and their Ig undergoes rearrangement.

5.1.2 Peripheral tolerance

It has been reported that some of these self-reactive T cells may escape into the periphery (Liu *et al.*, 1995). These “escaped” cells may be dangerous as they can potentially be recruited to give an autoimmune response. To monitor such potentially dangerous cells, a mechanism termed peripheral tolerance is in place to prevent these cells from orchestrating an autoimmune attack (Kisielow *et al.*, 1988; Walker *et al.*, 2002). Clones of cells that respond to self-antigen are generally

deleted preventing the proliferation of these cells in response to the antigen (McCaughy *et al.*, 2008). The key mechanisms that maintain tolerance in the periphery include clonal deletion, anergy, ignorance, and regulation. Most cells of the immune system require two signals for the activation of the cells; T cells for instance require a signal through the TCR as well as a second co-stimulatory activation signal(s). The absence of the second activation signal lead to the induction of cell death, induction of anergy (a state of unresponsiveness to antigen) and robust tolerance (Chen, 2004). T cells can differentiate into different subtypes: Th1 cells which are IFN γ producers and promote intracellular; Th2 cells which produce IL4 and IL5 which promote humoral immunity and allergic responses (Mosmann *et al.*, 2005). Another subset of T cells is the Th17 T cells which produce IL17 and are involved in the control of certain autoimmune or infectious diseases (Weaver *et al.*, 2006). Cell activation is delicately counterbalanced by the production of regulatory cells some of which appear to be inducible in the periphery; these regulatory T cells (T regs) another subset of T cells, are thought to be central to peripheral tolerance through their suppressive function (Bach, 2003). T regs suppress several cell types including CD4⁺ helper cells, CD8⁺ cytotoxic T lymphocytes, antibody production by B cells and antigen presenting (APC) function (Eddahri *et al.*, 2006; Mempel *et al.*, 2006; Taams *et al.*, 2000). In a subset of T regs known as CD25^{high}-FoxP3⁺ T regs, there is an upregulation of forkhead transcription factor (Foxp3) following activation, expression of FoxP3 does not always correlate with the suppressive function (Tran *et al.*, 2007). Another subset of T regs is the type 1 T regs (Tr1) that are suppressive through the production of high levels of IL10 and transforming grown factor beta (TGF β) (Groux *et al.*, 1997). Tr1 cells do not express Foxp3 (Vieira *et al.*, 2004). A third subset of T regs is known as Th3 cells, these are regulatory cells that are a result of conversion of naive CD4 T cells to inducible T regs by TGF β (Chen *et al.*, 2003). Tolerance interventions may

boost the numbers and functions of T regs and/or inhibit the activation of naive T cells by co-stimulatory blockage (St Clair *et al.*, 2007).

A breakdown in tolerance can lead to an immune response to an antigen that would otherwise not elicit an immune response. A breakdown in tolerance to self-antigen for example is an underlying thread in many autoimmune diseases; and allergic diseases involve an impaired tolerance to otherwise harmless molecules. In MS, breaking of tolerance to auto-antigens e.g. myelin basic proteins, is thought to be of pathogenic relevance in the clinical outcome (such as lesion distribution) of the disease (Greer *et al.*, 2008).

5.1.3 Tolerization strategies

Many strategies for induction of tolerance have been reported in naive immune cells including oral, nasal and intravenous route of immunization and high or low dose antigen. Memory T cells have less stringent activation requirements than naive cells with reduced and in some cases no co-stimulation, as such it can be difficult to induce tolerance in mice with memory T cells (Valujskikh, 2006). The intravenous (i.v.) route of antigen introduction for instance has been used to boost antibody responses and i.v. MOG stimulated a B cell response leading to the exacerbation of chronic EAE in marmosets (Genain *et al.*, 1996). The intravenous administration of antigen has most consistently induced antigen-specific tolerance in established cell-mediated autoimmunity or hypersensitivity. Tamura *et al* successfully induced immune tolerance to Factor VIII (FVIII) by continuous infusion of recombinant FVIII, without this most haemophilia patients may have limited alternative treatment (Tamura *et al.*, 2006). The main mechanisms involved in i.v. induction of tolerance are clonal deletion, induction of regulatory T cells and anergy of antigen-

specific T cells (Jacobs *et al.*, 1994). Improved variations to the i.v. method of tolerance induction have also been reported in animal models including cross-linking antigen (peptides) to splenocytes with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI) a cross-linking agent. Cross-linking of splenocytes with antigens induces an antigen-specific tolerance in the EAE model which led to prevention and treatment of disease in this model. The splenocytes-antigen linked tolerance induced was long-lasting and improved tolerance in comparison with soluble peptides (Turley *et al.*, 2007). Another method for improving tolerance induction is by the depletion of cell subsets prior to i.v. introduction of antigen. In the EAE model, it has previously been shown that reduction of cell subset pools (CD4 T cell depletion) prior to tolerance induction produced a robust unresponsiveness in established EAE disease (Pryce *et al.*, 2005). The reduction of cell pools prior to tolerance induction was used in this project as a method to try and improve the induction of tolerance to rhIFN β in the NABs model.

The depletion strategies used in this project include CD4 and CD20 depleting antibodies, and administration of mitoxantrone. These depletion strategies have previously been tested/trialed in EAE models and MS patients. Administration of CD4-specific monoclonal antibodies resulted in reduced EAE severity in EAE model (Pryce *et al.*, 2005); and in MS patients, treatment resulted in depletion of CD4 T cells, however beneficial clinical effects measured by the number of active lesions by MRI were lacking (van Oosten *et al.*, 1997).

Rituximab®, a humanized mouse monoclonal antibody against the human CD20 surface molecule, has been shown to reduce relapse rate by about 50% in relapsing-remitting MS (Bar-Or *et al.*, 2008). EAE severity was reduced in a transgenic EAE model treated with Rituximab® prior to immunization or at the onset of the clinical signs (Monson *et al.*, 2011). Mitoxantrone, another depletion

strategy that was investigated in this project, causes a reduction in CD4 and CD8 T cells, macrophages and B cells in the spleen (Piao *et al.*, 2007), and has been shown to significantly reduce the severity of acute phase EAE and also can prevent the onset of EAE if used pre-infection in a dose-dependent manner (Baker *et al.*, 1992). Clinical trials data also suggest efficacy of mitoxantrone in reducing relapses and slowing disease progression in relapsing-remitting and secondary progressive MS (Debouverie *et al.*, 2007).

All three depletion strategies were incorporated into the tolerization method to investigate methods of improving tolerance induction in the NAbs model.

5.2 Results

5.2.1 Induction of neutralizing antibody

Mice were immunized with recombinant human IFN β (Avonex®, Betaferon® or Rebif®) in CFA via the sub-cutaneous route (section 2.4); and sera/plasma samples collected (section 2.6) and tested in the neutralizing antibody assay (section 2.8). The 50% point between maximum and minimum log LCPS (luminescent counts per second) was determined and used to calculate the percentage inhibition; titres were also calculated for each sample tested (section 2.8.4). Naïve, unimmunized mice serum was used as a negative control.

To investigate the background levels of NAbs induction in the NAbs model, BALB/c mice (n=8) were immunized sub-cut with 20 μ g Betaferon® on day 0 and serum samples collected and tested in the NAbs assay on day 7. No NAbs were observed in these mice (figure 5.1). BALB/c mice (n=7) were immunized sub-cut with 20 μ g Betaferon® on days 0 and 7 and serum samples collected and tested in the NAbs assay on day 21. NAbs were observed in 5 of the 7 mice tested at this time point (figure 5.2). Table 5.1 is a representative graph of NAbs observed on day 35 in this project in mice immunized sub-cut with 20 μ g Betaferon® on days 0, 7 and 21.

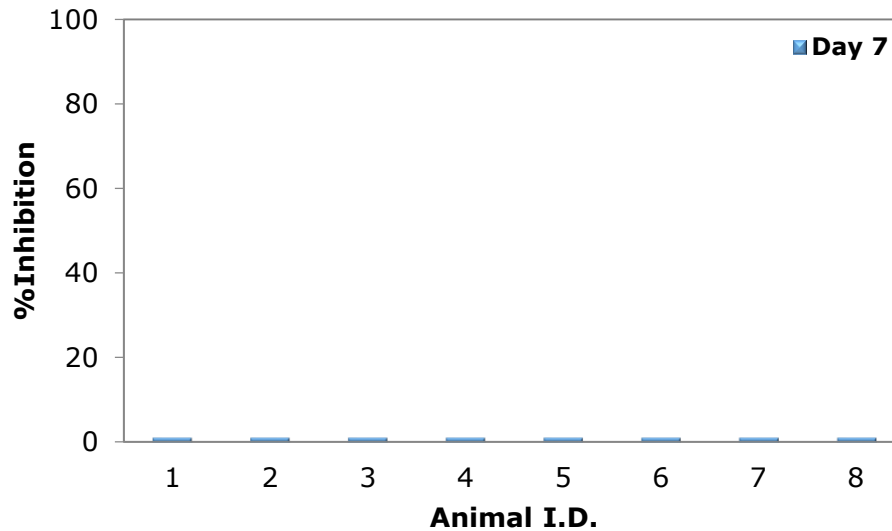


Figure 5.1: Neutralizing antibodies to Interferon-beta in BALB/c mice. BALB/c mice (n=8) were immunized sub-cut with 20 μ g Betaferon® in complete Freund's adjuvant on day 0 and serum samples collected and tested in the NAbs assay on day 7. %inhibition is the percentage of IFN β that was inhibited by the sample upon incubation. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. No NAbs to IFN β were observed in all test mice.

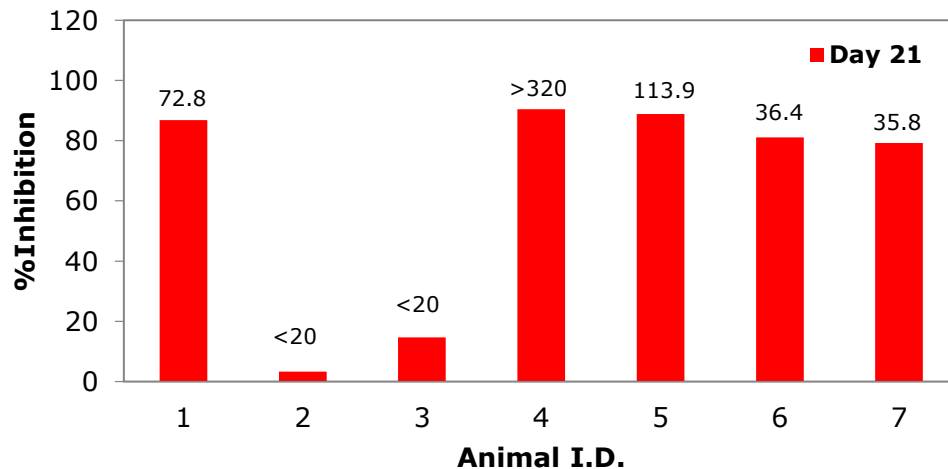


Figure 5.2: Neutralizing antibodies to Interferon-beta in BALB/c mice. BALB/c mice (n=7) were immunized sub-cut with 20 μ g Betaferon® in complete Freund's adjuvant on days 0 and 7; serum samples collected and tested in the NAbs assay on day 21. Naïve mouse serum was used as a control and was not immunized. %inhibition is the percentage of IFN β that was inhibited by the sample upon incubation. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. NAbs to IFN β were observed in 5 of the 7 test mice.

	20 µg		20 µg		20 µg	
	%Inhibition	Titre	%Inhibition	Titre	%Inhibition	Titre
1	74.8	232.1	27.6	<20	76.5	130.8
2	64.3	47.7	64.4	53.5	1.0	<20
3	50.2	<20	67.7	166.0	79.8	102.5
4	40.4	<20	15.6	<20	48.3	<20
5	73.1	118.4	71.8	75.9	49.8	<20

Table 5.1: Neutralizing antibodies to Interferon-beta in BALB/c mice. BALB/c mice (n=15) were immunized sub-cut with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; serum samples collected and tested in the NAbs assay on day 35. Naïve mouse serum was used as a control and were not immunized. %inhibition is the percentage of IFNβ that was inhibited by the sample upon incubation. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. NAbs to IFNβ were observed in 8 of the 15 test mice.

5.2.2 Effects of intravenous recombinant human interferon on NAbs induction

NAbs to rhIFNβ has been observed on day 35 in BALB/c mice immunized sub-cut with 20 µg Betaferon® on days 0, 7 and 21 (table 5.1). To investigate the effects of i.v. rhIFNβ on NAbs induction in the NAbs model, BALB/c mice (n=15) were first given 12.5 µg intravenous Betaferon® per mouse on day -8 and were then immunized sub-cut with 20 µg Betaferon® on days 0, 7 and 21 and samples were analyzed in a NAbs assay on Day 35 to investigate the development of NAbs. NAbs to rhIFNβ were observed in 6 of the 15 mice tested (table 5.2), in comparison with mice that were immunized sub-cut without i.v. administration of Betaferon® where an average of 50-70% of the samples were NAbs positive.

	20 µg		20 µg		20 µg	
	%Inhibition	Titre	%Inhibition	Titre	%Inhibition	Titre
1	74.6	39.6	40.9	<20	88.1	66.6
2	47.4	<20	90.2	130.6	26.4	<20
3	0	<20	7.2	<20	8.5	<20
4	17.3	<20	88.4	67.5	26.4	<20
5	73.9	42.5	73.4	37.3		

Table 5.2: Neutralizing antibodies to Interferon-beta in BALB/c mice. BALB/c mice (n=15) were given 12.5 µg Betaferon® intravenously on day -8 and immunized sub-cut with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; serum samples collected and tested in the NAbs assay on day 35. Naïve mouse serum was used as a control and were not immunized. %inhibition is the percentage of IFNβ that was inhibited by the sample upon incubation. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. NAbs to IFNβ were observed in 6 of the 15 test mice.

5.2.3 Effects of depletion and tolerance induction strategies on NAbs

BALB/c mice (n=40) were immunized sub-cut with 20 µg Betaferon® on days 0, 7 and 21 to induce NAbs to rhIFNβ, serum samples were tested in the NAbs assay on day 35 and NAbs to rhIFNβ were observed in these mice (figure 5.5). These mice were then depleted of immune cells by administration of anti-CD20 or anti-CD4 antibodies, or with mitoxantrone on day 38, control group had no depletion strategy. Anti-mouse CD20 antibody was administered i.v. at 250 µg/mouse, YTS191 CD4 depleting monoclonal antibody was administered i.p. at 200 µg/mouse and mitoxantrone administered i.p. at 2.5 mg/kg. All mice were given intravenous IFNβ (Rebif® at 12.5 µg/mouse) on day 52. The mice that were given mitoxantrone were poorly and had to be discontinued from the study. The other mice were finally re-challenged with sub-cut IFNβ (Betaferon® at 20 µg/mouse) on day 55 and all

mice culled on day 65 (figure 5.3). NAbs was observed in approximately half of all samples tested on day 35 (figure 5.4 and table 5.3).

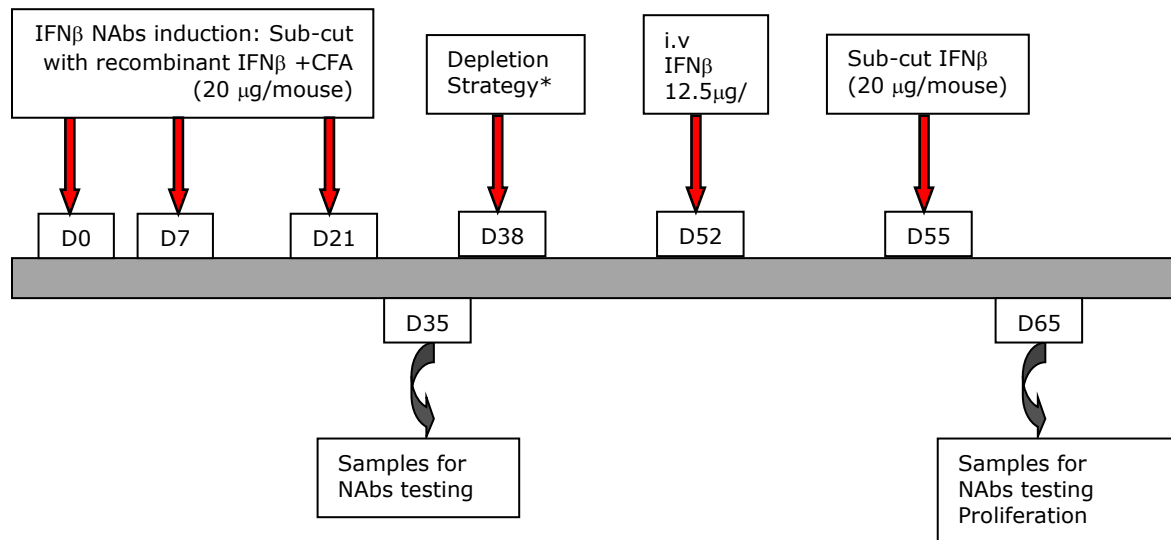


Figure 5.3: Timeline for immunization schedule for the administration of interferon-beta, depletion strategy and induction of tolerance in BALB/c mice. BALB/c mice (n=30) were immunized sub-cut with 20 μg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; immune cells were depleted on day 38 by administration of anti-CD20 antibody (n=10 at 250 μg/mouse, i.v.), anti-CD4 antibody (n=10 at 200 μg/mouse, i.p.) or no depletion (n=10). All mice were administered with i.v. Rebif® (at 12.5 μg/mouse) on day 52; all mice were finally given 20 μg Betaferon® sub-cut on day 55 and samples taken on days 35 and 65.

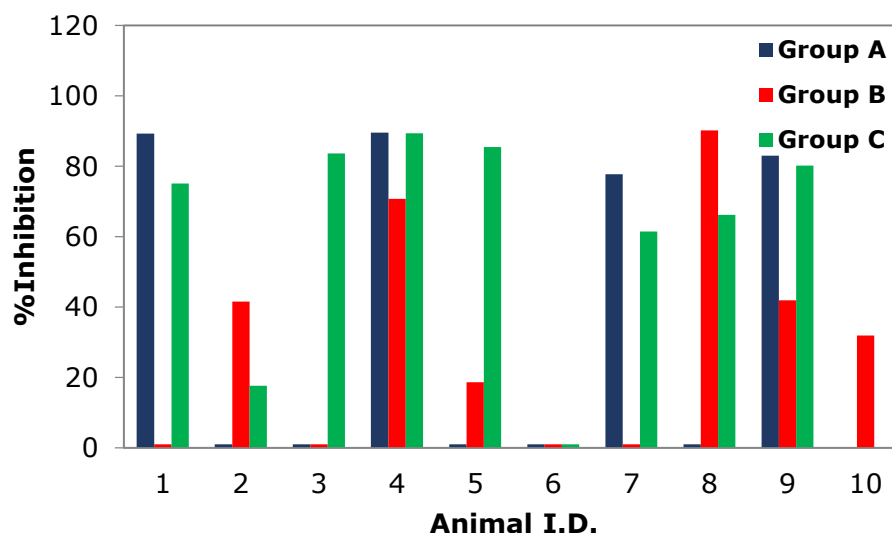


Figure 5.4: Neutralizing antibodies to Interferon-beta in BALB/c mice. BALB/c mice (n=30) were immunized sub-cut with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; serum samples collected and tested in the NAbs assay on day 35. %inhibition is the percentage of IFNβ that was inhibited by the sample upon incubation. NAbs to IFNβ were observed in 13 of the 30 test mice.

Titres	Positive	Negative
<20 (negative)	-	14
20-99	11	-
100-320	-	-
>320	2	-

Table 5.3: Neutralizing antibodies to Interferon-beta in BALB/c mice. BALB/c mice (n=30) were immunized sub-cut with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; serum samples collected and tested in the NAbs assay on day 35. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. NAbs to IFNβ were observed in 13 of the 30 test mice.

The samples taken on day 65 post tolerance induction were tested in the NAbs assay. 5 of the 10 mice in the anti-CD4 treated group were NAbs positive in

comparison with the control group (no depletion) and the anti-CD20 treated groups which had 3 of 10 mice NABs positive (figure 5.5 and table 5.4).

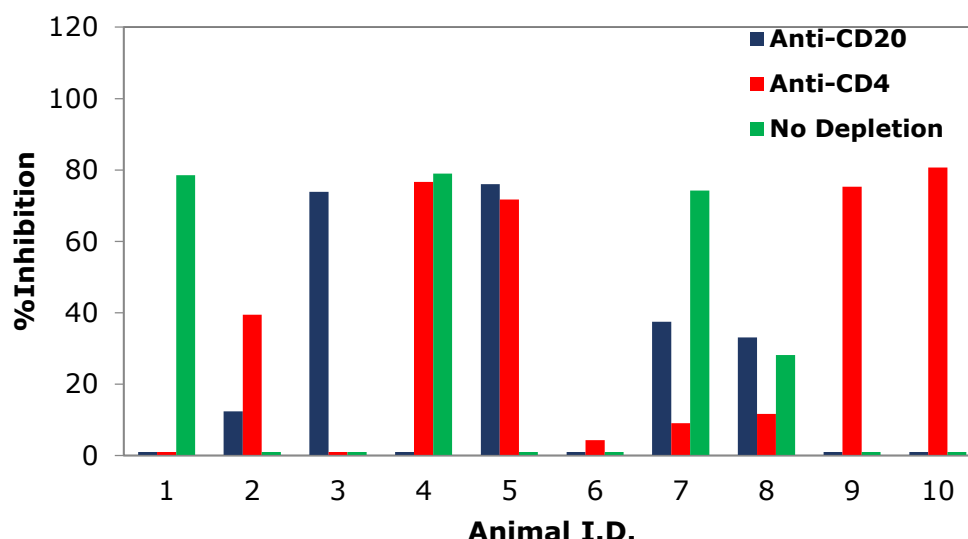


Figure 5.5: Neutralizing antibodies to Interferon-beta in BALB/c mice. BALB/c mice (n=30) were immunized sub-cut with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; immune cells were depleted on day 38 by administration of anti-CD20 antibody (n=10 at 250 µg/mouse, i.v.), anti-CD4 antibody (n=10 at 200 µg/mouse, i.p.) or no depletion (n=10). All mice were administered with i.v. Rebif® (at 12.5 µg/mouse) on day 52; all mice were finally given 20 µg Betaferon® sub-cut on day 55 and serum samples tested in the NABs assay on day 65. %inhibition is the percentage of IFNβ that was inhibited by the sample upon incubation. Titres less than 20 were deemed NABs negative and titres between 20 and >320 were deemed positive. 3 in the Control group and anti-CD20 treated group were NABs positive and 5 in the anti-CD4 treated group were NABs positive.

	Anti-CD20		Anti-CD4		No Depletion	
	%Inhibition	Titre	%Inhibition	Titre	%Inhibition	Titre
1	0	<20	0	<20	78.5	85.1
2	12.4	<20	39.5	<20	0	<20
3	73.9	45.3	0	<20	0	<20
4	0	<20	76.7	127.1	79.0	70.4
5	76.0	134.7	71.7	42.5	0	<20
6	0	<20	4.3	<20	0	<20
7	37.5	<20	9.1	<20	74.2	45.9
8	33.1	134.8	11.7	42.5	28.2	<20
9	0	<20	75.3	65.8	0	<20
10	0	<20	80.7	162.0	0	<20

Table 5.4: Neutralizing antibodies to Interferon-beta in BALB/c mice. Titres less than 20 were deemed NAbs negative and titres between 20 and >320 were deemed positive. 3 in the Control group and anti-CD20 treated group were NAbs positive and 5 in the anti-CD4 treated group were NAbs positive.

5.2.4 Proliferating cells and cytokine production

Mice were immunized sub-cut with recombinant human IFN β (Betaferon®) in CFA and splenocytes collected and tested in the proliferation assay (section 2.7.4). Table 5.5 is a summary of results observed in this section.

Immunization schedule	Cytokine results		Figure	Summary
	CD4 IFN γ	CD8 IFN γ		
Multiple immunization only				
Sub-cut Betaferon	++ (Day 7)	-	5.6	Significant IFN γ and IL-17 in CD4 and CD8 T cells
			5.7	
Intravenous administration				
I.v. Betaferon (12.5-750 μ g) post multiple immunizations	+	+	5.8	CD4 T cell proliferation reduced. IFN γ unaltered in CD4 and CD8 T cells
			5.9	
I.v. Betaferon (12.5 μ g) post immunization	+	+	5.10	IFN γ in 25% of test samples
I.v. Betaferon (12.5 μ g) post multiple immunizations	+	++	5.11	IFN γ more in CD8 than CD4 T cells
I.v. Betaferon (12.5 μ g) pre and post multiple immunizations	+	+	5.12	IFN γ in CD8 and CD4 T cells in 80% of test samples
			5.13	
Depletion Strategies				
Multiple immunization + anti-CD4 depletion + i.v. Betaferon	+	+	5.14	IFN γ more in CD8 than CD4 T cells
Multiple immunization + anti-CD20 depletion + i.v. Betaferon	+	+	5.15	IFN γ more in CD8 than CD4 T cells
Multiple immunization + no depletion + i.v. Betaferon	+	+	5.16	IFN γ more in CD8 than CD4 T cells

Table 5.5: Summary of Cytokine responses observed following different immunization schedules.

To investigate the background levels of proliferation and cytokine production in CD4 and CD8 T cells in the NAbs model, BALB/c mice (n=15) were immunized sub-cut with 20 μ g Betaferon® on day 0, 7 and 21, splenocytes were collected and tested in the proliferation assay 7 days post each immunization (days 7, 14 and 28). rhIFN β specific proliferation and IFN γ production were observed in CD4 T cells in mice immunized on day 0, 7 and 21 (figure 5.7). Figure 5.6 shows representative plots

observed. CD4 T cells were proliferating in response to rhIFN β and were producing IFN γ at all-time points investigated. Proliferation was also observed in CD8 T cells but no rhIFN β -specific IFN γ production were observed in these subset of cells. IL-17 production was also investigated in CD4 and CD8 proliferating T cells (figure 5.8). CD4 and CD8 T cells were proliferating and producing IL-17 in response to rhIFN β following day 0 immunization. The rhIFN β specific IL-17 responses appear to have been lost or reduced following subsequent immunizations.

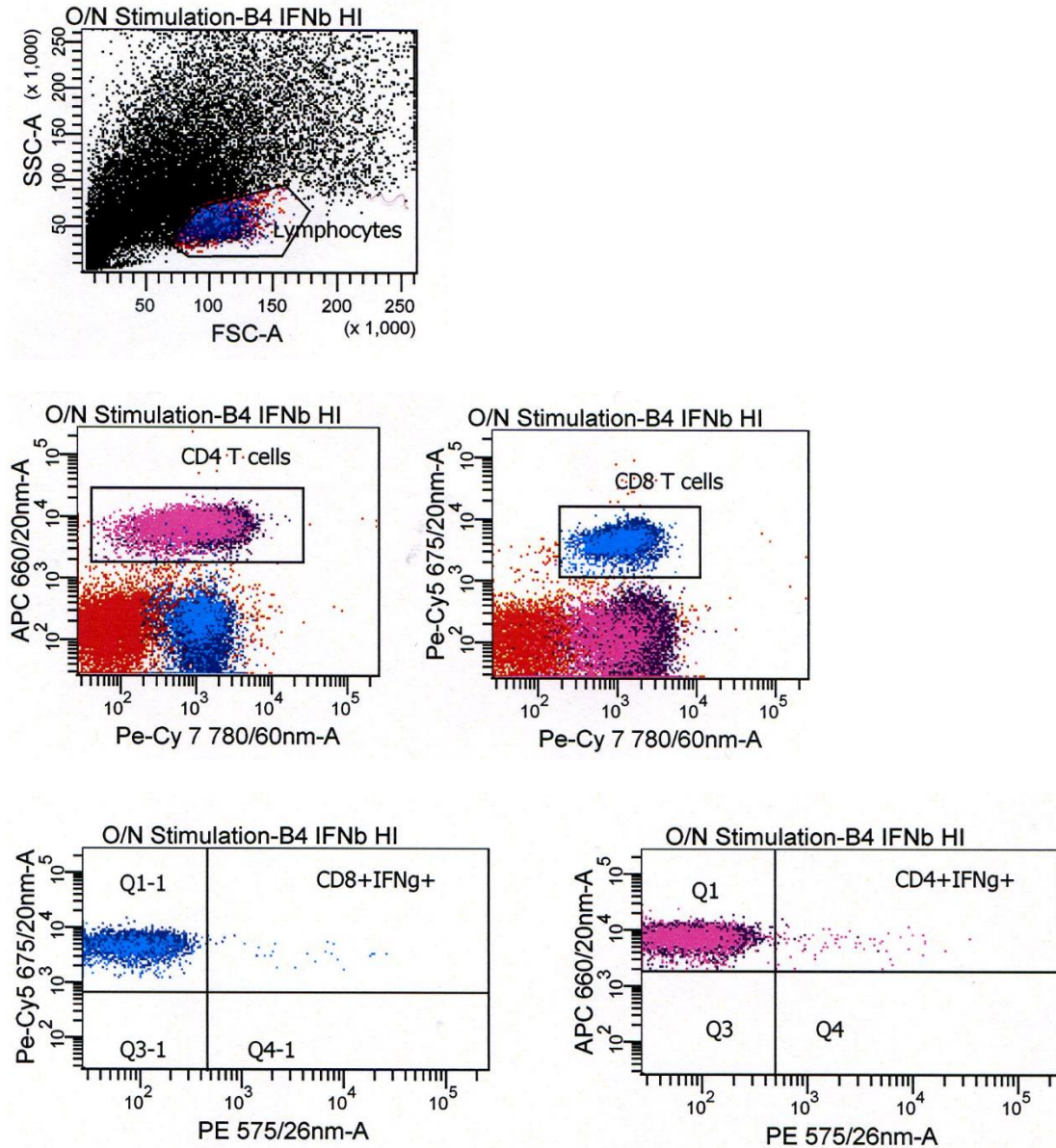


Figure 5.6: CD4 and CD8 T cell IFN γ production. Flow cytometry analysis of CD4 and CD8 T cell population in NAb mice. Splenocytes were stained for CD4 or CD8 T cell markers; CD4 and CD8 T cells were gated in the lymphoid population to determine proportions producing IFN γ in response to rhIFN β .

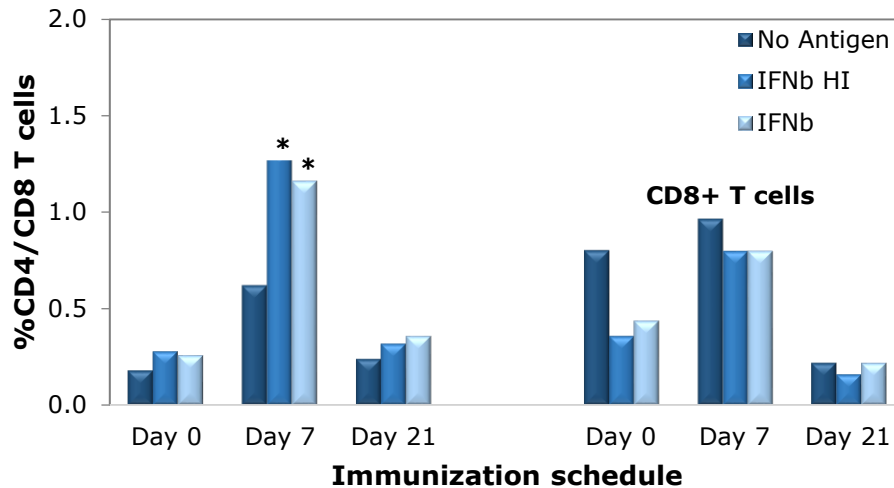


Figure 5.7: Interferon-beta specific proliferation and interferon-gamma production in BALB/c mice. BALB/c mice (n=15) were immunized sub-cut with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; splenocytes were collected and tested in the proliferation assay 7 days post each immunization. Proliferating CD4 and CD8 T cells were gated and proportions producing IFN γ were analysed for each test mice. The graph shows an average of the test mice at each time immunization time point. Proliferating CD4 T cells were producing IFN γ on days 0, 7 and 21 in response to rhIFN β ; day 7 showing the most marked response. Significance of P<0.05 (*), P<0.01(**) or P<0.001(***) in comparison with no antigen control.

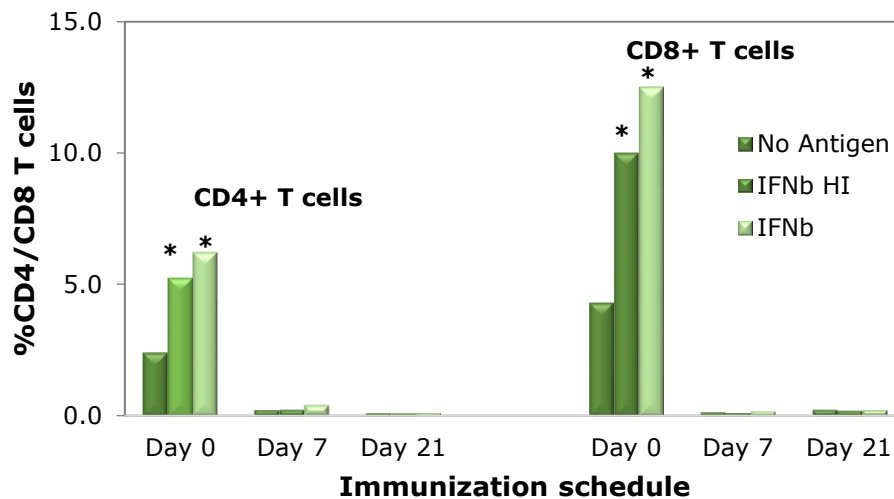


Figure 5.8: Interferon-beta specific proliferation and interleukin-17 production in BALB/c mice. BALB/c mice (n=15) were immunized sub-cut with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; splenocytes were collected and tested in the proliferation assay 7 days post each immunization. Proliferating CD4 and CD8 T cells were gated and proportions producing IL-17 were analysed for each test mice. The graph shows an average of the test mice at each time immunization time point. Proliferating CD4 and CD8 T cells were producing IL-17 on day 0 in response to both heat-inactivated (IFN β HI) and the natural IFN β . Significance of P<0.05 (*), P<0.01(**) or P<0.001(***) in comparison with no antigen control.

5.2.5 Effects of intravenous recombinant human interferon on proliferating T cells

rhIFN β specific proliferation and cytokine production were observed in CD4 and CD8 T cells from mice immunized with Betaferon® on days 0, 7 and 21. To investigate the influence of i.v. rhIFN β on these cells in the NAbs model, BALB/c mice were immunized sub-cut with 20 μ g Betaferon® on days 0, 7 and 21 and given i.v. Rebif® at various concentrations (12.5 – 750 μ g/mouse) on day 39. The control group was not given i.v. Rebif®. A large proportion of CD4 T cells in the control group were proliferating in comparison with the test groups (figure 5.9). rhIFN β specific IFN γ production in proliferating CD4 T cells were observed in all groups including the control group (figure 5.10).

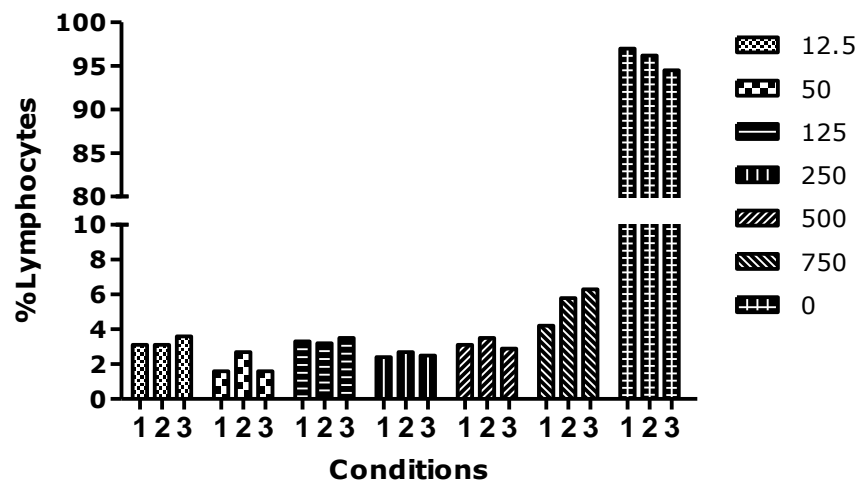


Figure 5.9: CD4 T cell proliferation in BALB/c mice. BALB/c mice (n=42) were immunized with 20 μ g Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; then given i.v. Rebif® at various concentrations (0-750 μ g/mouse) on day 39 and finally immunized 20 μ g/mouse sub-cut Betaferon® on day 42. Splenocytes were collected and tested in the proliferation assay on day 52. Proliferating CD4 and CD8 T cells were gated and analysed for each pooled group. In the control group (no i.v. Rebif®), a larger proportion of CD4 T cells were proliferating in comparison with the groups given i.v. Rebif®. Conditions: 1=No Antigen, 2=IFN β heat-inactivated and 3= IFN β .

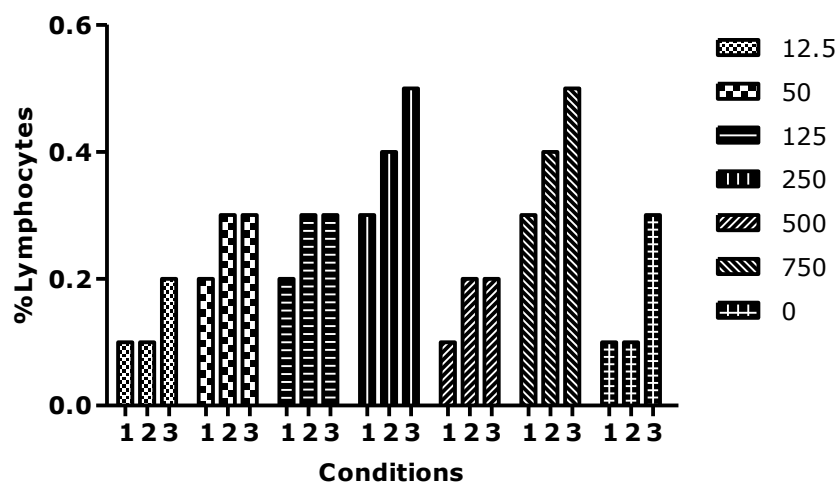
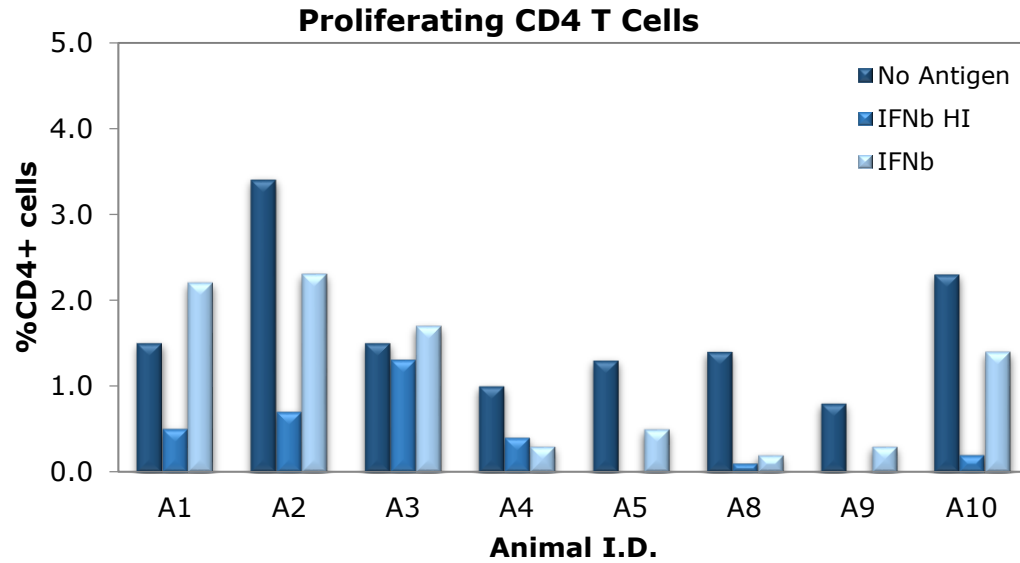


Figure 5.10: Interferon-beta specific CD4 T cell proliferation and IFN γ production in BALB/c mice. BALB/c mice (n=42) were immunized with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; then given i.v. Rebif® at various concentrations (0-750 µg/mouse) on day 39 and finally immunized 20 µg/mouse sub-cut Betaferon® on day 42. Splenocytes were collected and tested in the proliferation assay on day 52. Proliferating CD4 and CD8 T cells were gated and IFN γ production analysed for each pooled group. rhIFN β specific IFN γ production was observed in the groups that received i.v. Rebif and also in the control group. Conditions: 1=No Antigen, 2=IFN β heat-inactivated and 3= IFN β .

To further investigate the effects of intravenous administration of rhIFN β on proliferative responses of CD4 and CD8 T cells, BALB/c mice (n=16) were immunized sub-cut with 20 µg Betaferon® on day 0, 7 and 21. All mice were given i.v Rebif® at 12.5 µg/mouse on day 37 and half the mice (n=8) were culled and splenocytes tested in the proliferation assay on day 42. CD4 and CD8 T cells proliferative responses were observed in 2 of these 8 test mice (figure 5.11). The other half (n=8) were immunized sub-cut with 20 µg/mouse Betaferon® on day 40 to see if further immunization would affect the outcome of the T cells responses. Splenocytes were collected and tested in the proliferation assay on day 50; CD4 T cell responses were observed in 1 test mouse and 5 test mice showed CD8 T cell proliferative responses to rhIFN β (figure 5.12), these responses did not reach a significant level.

A)



B)

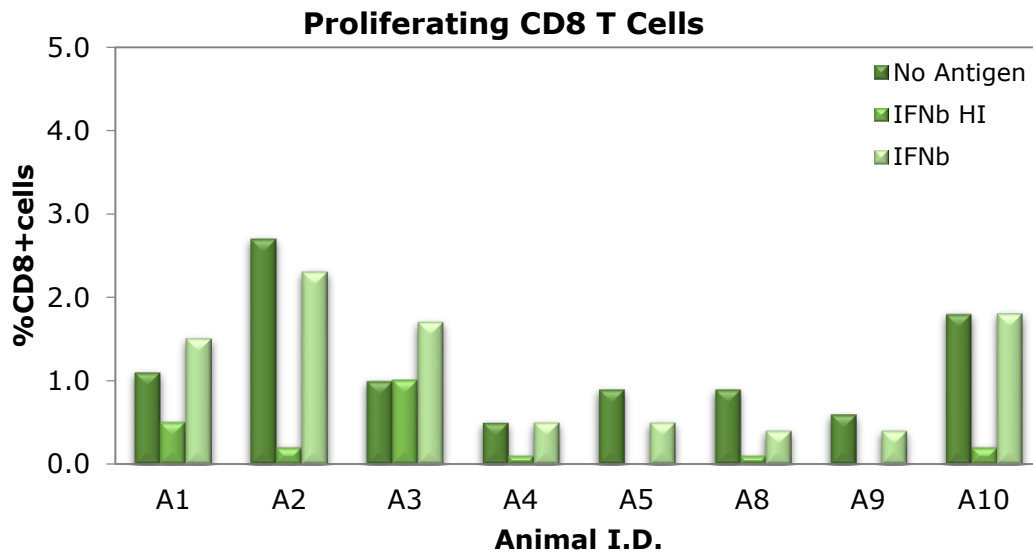
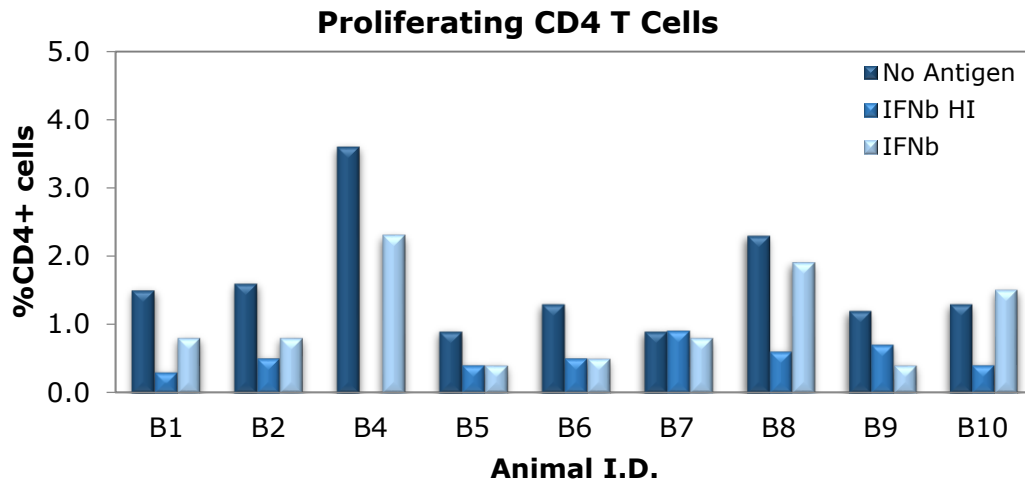


Figure 5.11: Interferon-beta specific proliferation in BALB/c mice. BALB/c mice (n=8) were immunized sub-cut with 20 μ g Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; then given i.v. Rebif® at 12.5 μ g/mouse on day 37. Splenocytes were collected and tested in the proliferation assay on day 42. Proliferating CD4 and CD8 T cells were gated and analysed for each test mouse. rhIFN β specific CD4 and CD8 T cell proliferation was observed in 2 of the 8 mice tested. A) CD4 T cell proliferation, B) CD8 T cell proliferation.

A)



B)

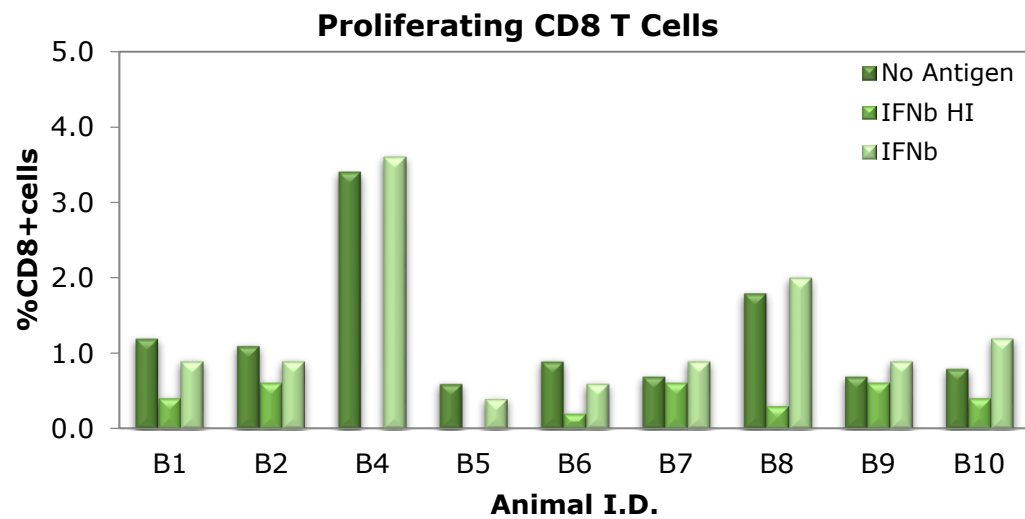
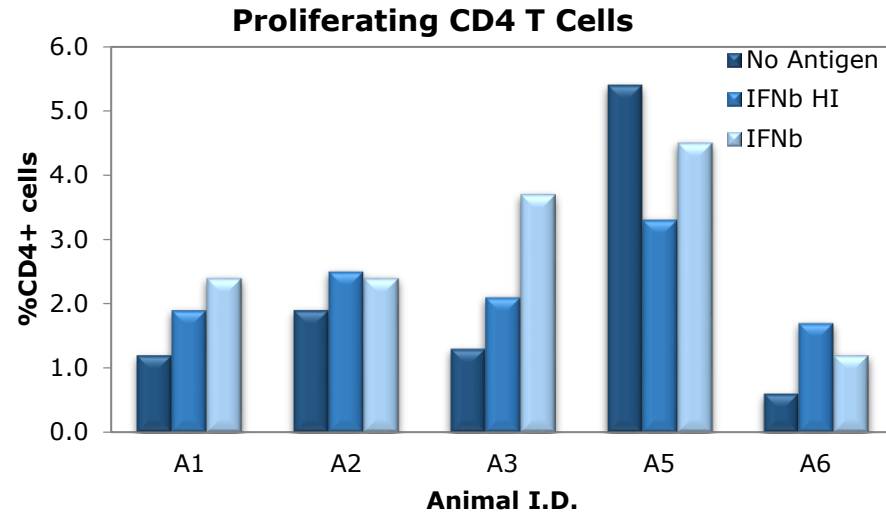


Figure 5.12: Interferon-beta specific proliferation in BALB/c mice. BALB/c mice (n=8) were immunized sub-cut with 20 μ g Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; then given i.v. Rebif® at 12.5 μ g/mouse on day 37 and finally immunized 20 μ g/mouse sub-cut Betaferon® on day 40. Splenocytes were collected and tested in the proliferation assay on day 50. Proliferating CD4 and CD8 T cells were gated and analysed for each test mouse. rhIFN β specific CD4 T cell proliferation was observed in 1 test mouse and CD8 proliferation observed in 5 of the 8 test mice. A) CD4 T cell proliferation, B) CD8 T cell proliferation.

To investigate further the effect of i.v. rhIFN β on proliferating cells, BALB/c mice (n=10) were first given 12.5 μ g intravenous Betaferon® per mouse on day -8 and immunized sub-cut with 20 μ g/mouse Betaferon® on days 0, 7 and 21. One group was given 12.5 μ g i.v Rebif® (group A) and the other group was given 125 μ g i.v. Rebif® (group B) on day 39 and both groups were rechallenged with 20 μ g/mouse IFN β (Betaferon®) sub-cut on day 43. In group A, 4 of the test mice showed CD4 and CD8 T cell responses specific for rhIFN β (figure 5.13). Similar results were observed in Group B (figure 5.14). Increasing the amount of Rebif® given i.v. did not appear to dramatically affect the numbers with CD4 and CD8 T cell proliferation responses once they have been established.

A)



B)

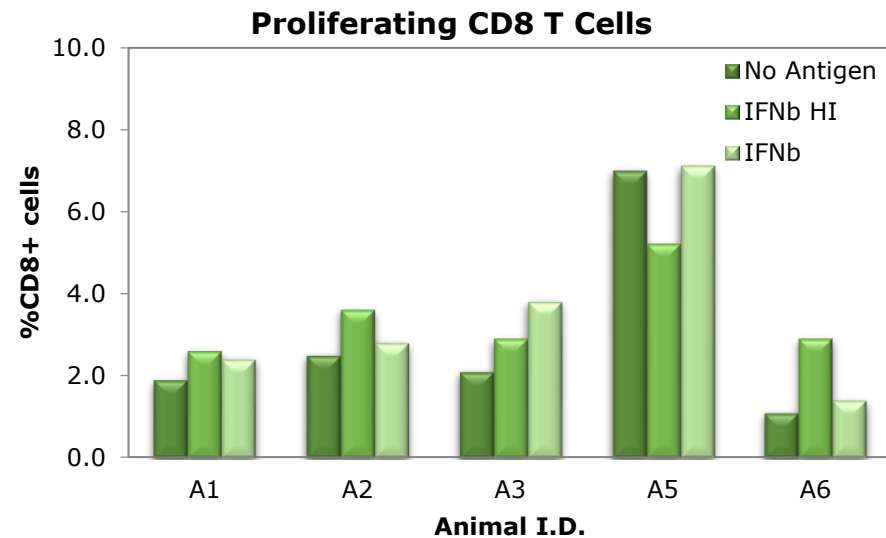
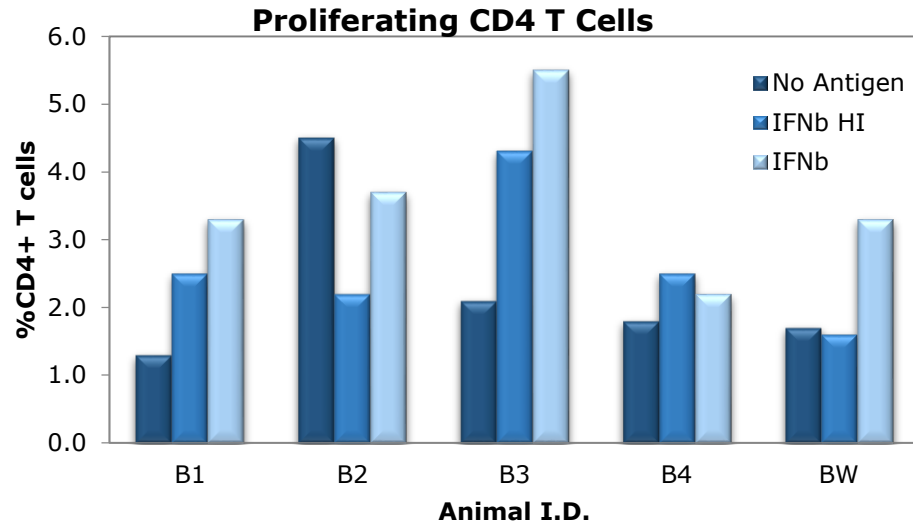


Figure 5.13: Interferon-beta specific T cell proliferation in BALB/c mice. BALB/c mice (n=5) were given 12.5 µg intravenous Betaferon® per mouse on day - 8 and immunized with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; then given i.v. Rebif® at 125 µg/mouse on day 39 and finally immunized with 20 µg/mouse sub-cut Betaferon® on day 43. Splenocytes were collected and tested in the proliferation assay on day 53. Proliferating CD4 and CD8 T cells were gated and analysed for each test sample. rhIFNβ specific CD4 and CD8 T cell proliferation were observed in 4 of the 5 test samples analysed. A) CD4 T cells proliferation, B) CD8 T cells proliferation.

A)



B)

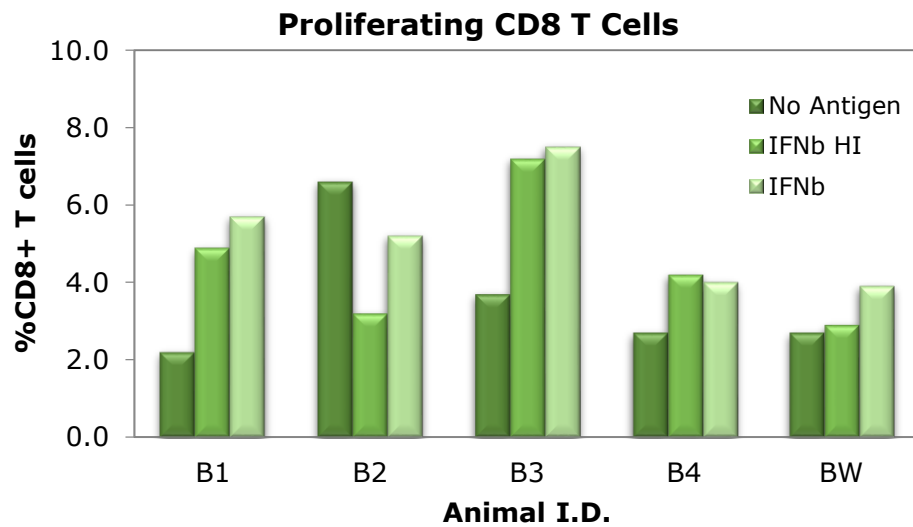
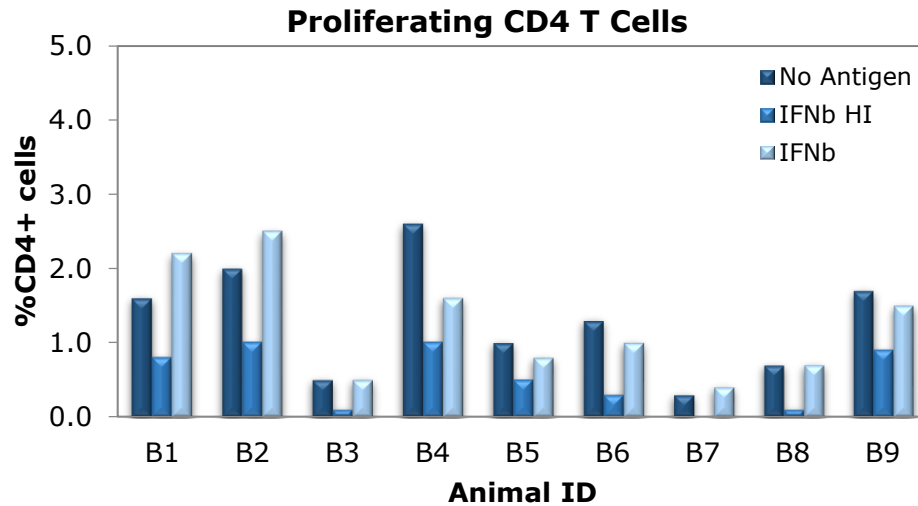


Figure 5.14: Interferon-beta specific T cell proliferation in BALB/c mice. BALB/c mice (n=5) were given 12.5 μ g intravenous Betaferon® per mouse on day - 8 and immunized with 20 μ g Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; then given i.v. Rebif® at 12.5 μ g/mouse on day 39 and finally immunized with 20 μ g/mouse sub-cut Betaferon® on day 43. Splenocytes were collected and tested in the proliferation assay on day 53. Proliferating CD4 and CD8 T cells were gated and analysed for each test sample. rhIFN β specific CD4 and CD8 T cell proliferation were observed in 4 of the 5 test samples analysed. A) CD4 T cells proliferation, B) CD8 T cells proliferation.

5.2.6 Effects of depletion and tolerance induction strategies on proliferating T cells.

BALB/c mice were immunized with 20 µg Betaferon® on days 0, 7 and 21 to induce NAbs to rhIFNβ. These mice were then depleted of immune cells by administration of anti-CD20 or anti-CD4 antibodies, or with mitoxantrone on day 38, control group had no depletion strategy. Anti-mouse CD20 antibody was injected i.v. at 250 µg/mouse, YTS191 CD4 depleting monoclonal antibody was injected i.p. at 200 µg/mouse and mitoxantrone injected i.p. at 2.5 mg/kg (section 2.3). All mice were given intravenous IFNβ (Rebif® at 12.5 µg/mouse) on day 52. The mice that were given mitoxantrone did not tolerate the drug and exhibited sickness behavior and had to be discontinued from the study. The other mice were finally rechallenged with sub-cut IFNβ (Betaferon® at 20 µg/mouse) on day 55 and all mice culled on day 65 and tested in the proliferation assay. In the anti-CD4 depletion group, CD4 T cell responses were observed in 2 of the 9 test mice and CD8 T cells responses specific for rhIFNβ were observed in 7 of the 9 test mice (figure 5.15). 5 of the 9 test mice in the group with anti-CD20 depletion strategy showed CD4 and CD8 T cell responses specific to rhIFNβ (figure 5.16). In the control group with no depletion strategy rhIFNβ specific CD8 T cell responses were observed in all test mice and 6 showed CD4 T cell proliferation in response to rhIFNβ (figure 5.17).

A)



B)

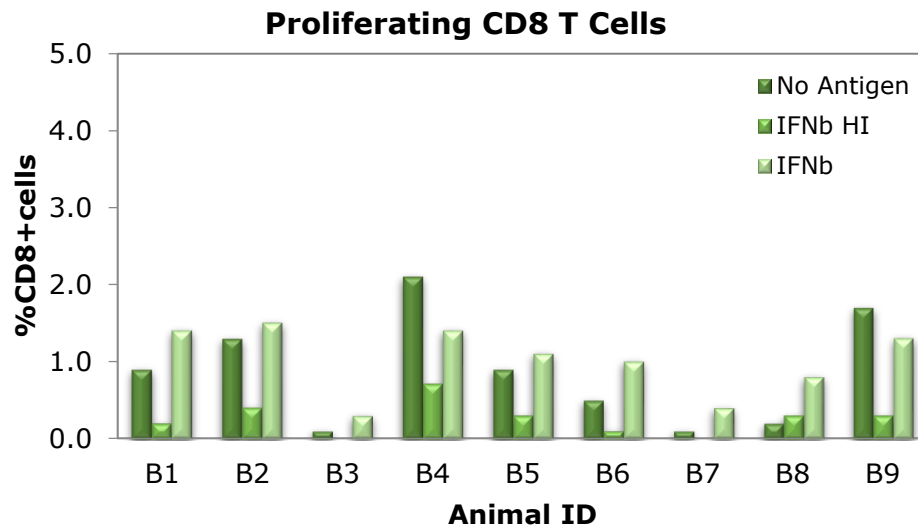
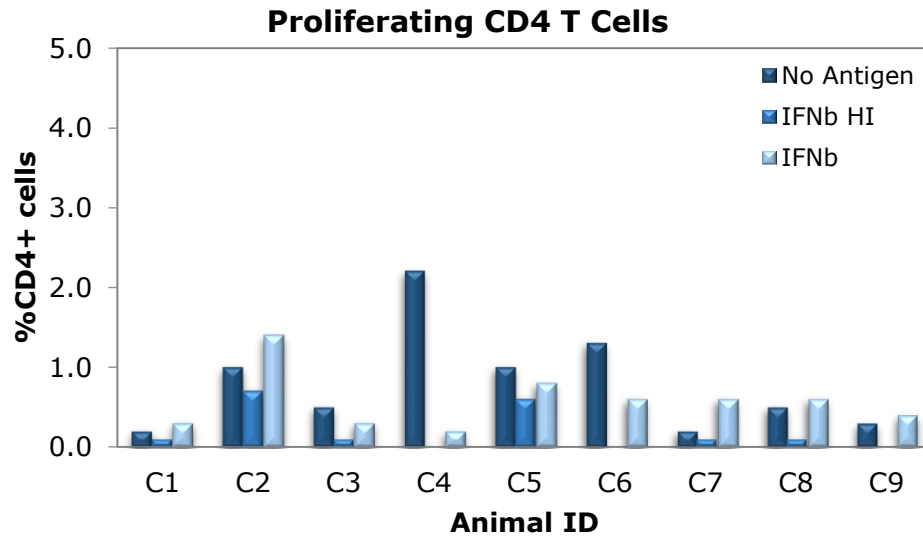


Figure 5.15: Interferon-beta specific T cell proliferation in BALB/c mice. BALB/c mice (n=10) were immunized with 20 μ g Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; immune cells were depleted on day 38 by administration of anti-CD4 antibody at 200 μ g/mouse, i.p. All mice were administered with i.v. Rebif® (at 12.5 μ g/mouse) on day 52; all mice were finally given 20 μ g Betaferon® sub-cut on day 55 and splenocytes tested in the proliferation assay on day 65. rhIFN β specific CD4 and CD8 T cell proliferation were observed in 2 (CD4 T cells) and in 7 (CD8 T cells) of the test samples analysed. A) CD4 T cells proliferation, B) CD8 T cells proliferation.

A)



B)

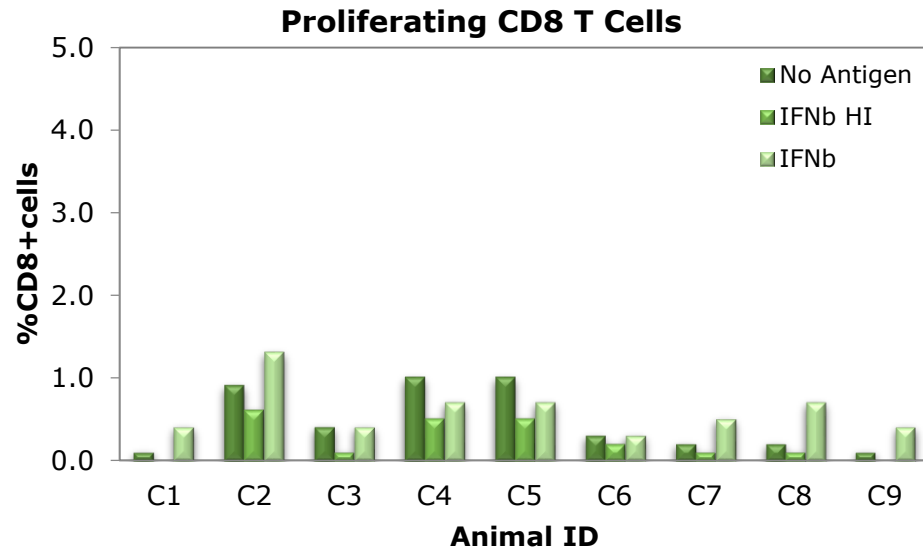
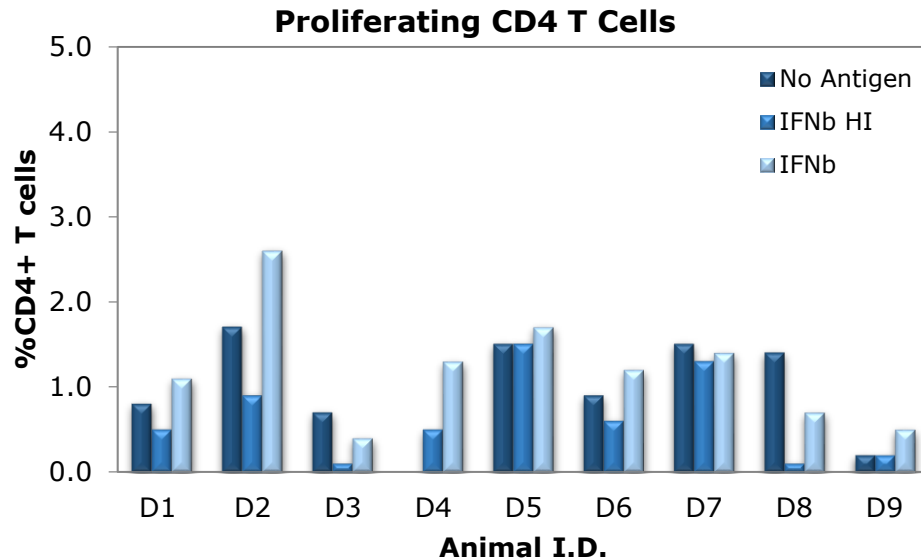


Figure 5.16: Interferon-beta specific T cell proliferation in BALB/c mice. BALB/c mice (n=10) were immunized with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; immune cells were depleted on day 38 by administration of anti-CD20 antibody at 250 µg/mouse, i.v. All mice were administered with i.v. Rebif® (at 12.5 µg/mouse) on day 52; all mice were finally given 20 µg Betaferon® sub-cut on day 55 and splenocytes tested in the proliferation assay on day 65. rhIFNβ specific CD4 and CD8 T cell proliferation were observed in 1 (CD4 T cells) and in 4 (CD8 T cells) of the test samples analysed. A) CD4 T cells proliferation, B) CD8 T cells proliferation.

A)



B)

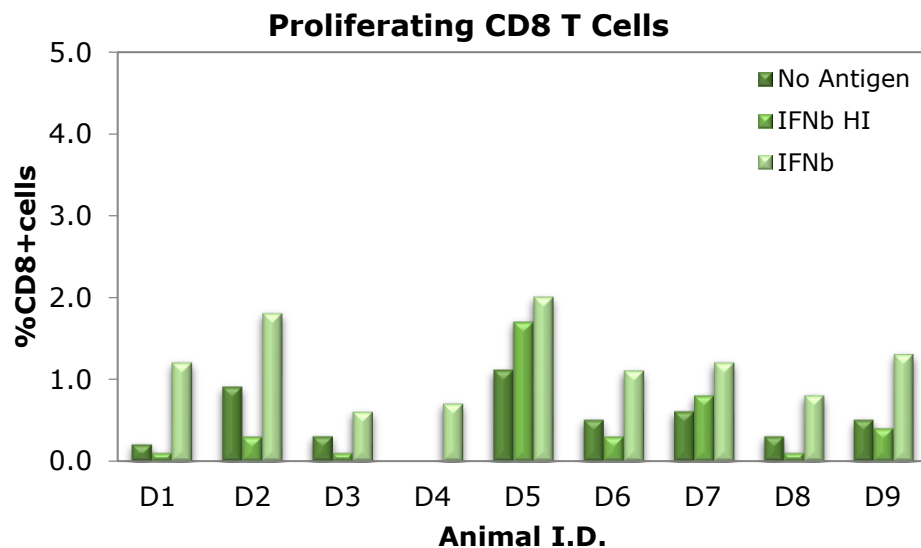


Figure 5.17: Interferon-beta specific T cell proliferation in BALB/c mice. BALB/c mice (n=10) were immunized with 20 µg Betaferon® in complete Freund's adjuvant on days 0, 7 and 21; no depletion strategy administered on day 38. All mice were administered with i.v. Rebif® (at 12.5 µg/mouse) on day 52; all mice were finally given 20 µg Betaferon® sub-cut on day 55 and splenocytes tested in the proliferation assay on day 65. rhIFNβ specific CD4 and CD8 T cell proliferation were observed in 5 (CD4 T cells) and in 9 (CD8 T cells) of the test samples analysed. A) CD4 T cells proliferation, B) CD8 T cells proliferation.

5.3 Discussion

NAbs to recombinant human interferon beta has been successfully established in the BALB/c mice and was termed the NAb model. This chapter details the strategies investigated in this model to induce tolerance to rhIFN β . The strategies investigated were depleting immune cells followed by re-introduction of rhIFN β via the intravenous route in the NAb model. NAb to rhIFN β and proliferative responses of CD4 and CD8 T cells were used as indicators for tolerance induction. This chapter demonstrates that a combination of depleting immune cells and i.v. re-introduction of rhIFN β in the NAb model reduced the incidence of NAb and CD4 and CD8 T cell responders in the combination strategy group.

In the NAb model, NAb to rhIFN β was established by sub-cut administration of the protein and rhIFN β -specific CD4 and CD8 T cells proliferative responses and cytokine (IFN γ and IL-17) production were also detected at the time of NAb formation. Intravenous administration of antigen to naïve cells favours the induction of tolerance to the antigen. In my project, i.v. rhIFN β in naïve BALB/c mice resulted in a reduction in the incidence of NAb. On the other hand, i.v. rhIFN β in the early phase of established NAb model did not appear to affect the incidence of NAb. It has been shown that tolerance induction by intravenous administration of antigen leads to an increase in the IgG levels (Verbeek *et al.*, 2007). In the NAb model, it is most likely that prior immunization with rhIFN β results in the development of mature rhIFN β -specific B cell and intravenous administration of rhIFN β leads to relocation of the antigen to the spleen where the primed B cells can rapidly respond. T cell responses were observed in the NAb model, intravenous administration of rhIFN β led to a reduction of 70-80% in rhIFN β -specific proliferative CD4 T cell responses in this model.

In established NAb model, giving i.v. rhIFN β prior to induction of NAb did not appear to affect the outcome of CD4 and CD8 T cell rhIFN β -specific responses in these mice. It could be that the amount of protein given was too low to establish tolerance. These mice were given a second round of i.v. rhIFN β at a high and low dose but this did not reduce the incidence of established T cell responses. In an adoptive transfer model, i.v. introduction of antigen resulted in an induction in tolerance, with high dose antigen inducing tolerance quicker than low dose antigen (Hayashi et. al, 2002). In my project, i.v. rhIFN β in established NAb model led to a reduction in antigen-specific proliferative responses by CD4 and CD8 T cells. Tolerance induction was not affected by the dose of antigen given as a range of low to high i.v. antigen resulted in reduction in CD4 and CD8 T cell proliferative responses.

The depletion strategies used in this project include CD4 and CD20 depleting antibodies, and administration of mitoxantrone. All 3 depletion strategies reduced cell pools when administered in naïve BALB/c mice; anti-CD4 antibodies significantly reduced CD4 T cells in spleen and blood, anti-CD20 antibody significantly reduced B cells in spleen and blood, and mitoxantrone significantly reduced CD4 and CD8 T cells in spleen and reduced CD4 T cells and B cells in the blood (discussed in chapter 4). All three depletion strategies were incorporated into the tolerization method to investigate methods of improving tolerance induction in the NAb model. Depletion of immune cells generally leads to a reconstitution of cell pools and during this window, it is hoped that the naïve pool can be orchestrated or influenced regarding a specific antigen by the route of administration of the antigen. Once NAb was established, the immune cells were depleted and rhIFN β re-introduced intravenously. The average incidence of NAb in the NAb model is between 50 and 100% and depletion of CD4+ and CD20+ cells prior to intravenous administration of rhIFN β led to a reduction in the incidence of NAb to between 30 and 50%.

Intravenous administration of rhIFN β alone reduced the incidence of NAbs to 30%. It appears that intravenous administration of the antigen led to the reduction in NAbs incidence and depletion of the cell subsets did not significantly affect the outcome of the incidence of NAbs. Depletion of cells subsets however appeared to influence T cell responses observed. In the non-depleted control group, more animals were proliferating in response to rhIFN β , though none reached significance. In the group depleted of CD4 T cell, there was an increase of CD8 T cell responses; this could be as a result of a reduction in mature CD4 T cells and the naïve pool being slowly replenished while the CD8 T cell mature pool was primed and ready to respond to the antigen. In the CD20 depleted group, reduction in CD4 and CD8 T cell responses observed could be due to the loss of B cells which have co-stimulatory molecules for T cell activation.

In the NAbs model, intravenous rhIFN β can induce tolerance in established T cell response. This is most likely as a result of apoptosis of memory T cells due to exposure to antigen without the appropriate co-stimulatory signals required. Incidence of NAbs was reduced by intravenous antigen. Depletion of immune cell pools prior to intravenous antigen reduced the incidence of NAbs and also reduced the numbers of CD4 and CD8 T cell antigen-specific responders. The depletion and tolerization strategy used in the NAbs model here could be applied to NAbs positive patients where the ideal clinical outcome would be an increase or prolonging in the efficacy of IFN β treatment in these patients. It is hoped that the regeneration and reprogramming of the immune system will lead to a regeneration of the natural regulatory events that curb autoimmune cells in the MS setting. Once the mechanism of a reconstituting immune system is better understood, we may be able to intervene and re-educate the immune system, which may pose a treatment strategy for autoimmune diseases like MS.

Chapter 6

Experimental autoimmune encephalomyelitis and depletion strategy

6.1 Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative disease and the major cause of neurological disability in young adults (Noseworthy *et al.*, 2000). The immune system is thought to be a key player in the progression of the disease (Bruck, 2005); this is supported by the current immunosuppressive and anti-inflammatory therapies that slow progression of disease and reduce clinical symptoms (Menge. *et al.*, 2008).

To date there are no definitive treatments which cure MS, but there are several therapies for reducing relapse rate and severity of clinical outcome of disease. These include Interferon-beta, mitoxantrone and several monoclonal antibodies including anti-CD20 and anti-CD52.

6.1.1 Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a widely used autoimmune model of MS. EAE can be induced by immunization of EAE models with several central nervous system (CNS) peptides such as myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein 1 (PLP1); and by spinal cord homogenates (Baker *et al.*, 1990b).

EAE is thought to be a T cell mediated disease as EAE can be induced by adoptive transfer of reactive EAE T cells (Pryce *et al.*, 2005). T helper 1 cells (Th1) cells have long been regarded as the mediators, but recently T helper 17 cells (Th17) have

also been shown to play an important role (Batoulis *et al.*, 2010). However, recent evidence highlights the importance of B cells in the development of EAE and MS. Autoantibodies have been detected in the cerebrospinal fluid of many MS patients, these antibodies have been against a number of antigens (Kanter *et al.*, 2006). Mature B cells have been found to cross the blood brain barrier in MS patients and may undergo restimulation within lesions (Magliozzi *et al.*, 2007). Abnormal B cell responses to cytokines have also been reported in MS patients (Smith-Jensen *et al.*, 2000). B cells may contribute to the development of EAE by the production of auto-reactive antibodies and by the presentation of antibodies to T cells.

Depletion of B cells with Rituximab (a humanized mouse monoclonal antibody against human CD20 surface molecule) has shown that B cells are pivotal for the development of EAE in a number of mouse models (Monson *et al.*, 2011). Rituximab has been shown to reduce relapse rate in RR-MS patients (Hauser *et al.*, 2008).

EAE models have helped in the development of several drugs for the treatment of MS including Glatiramer acetate (GA) (Arnon *et al.*, 1989), Natalizumab (Yednock *et al.*, 1992) and mitoxantrone (Piao *et al.*, 2007). The EAE model however does not predict clinical outcomes or toxicity in humans; as such the real strength of the EAE model is a pre-screen for potential drug treatments for MS (Steinman *et al.*, 2006).

The mouse is the most common specie used in EAE studies; other species have also been successfully used including rats, guinea pigs and marmosets. The type of EAE disease developed and the pathology observed depends on the strain of mouse used and also the antigen used to induce the disease. For instance in the Lewis rat, a single acute phase disease is observed and in the C57/BL6 mice acute progressive disease is observed. In the Biozzi antibody high (ABH) mouse, immunization with whole spinal cord homogenate in complete Freund's adjuvant leads to a chronic relapsing-remitting disease with secondary progression (Baker *et al.*, 1990b), when

this strain is immunized with myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide, a chronic progressive disease with little or no remission is observed (Amor *et al.*, 2005). The disease phenotype observed in the ABH mouse (relapsing-remitting with secondary progression) are similar to the types of disease seen in most MS patients; this indicates that the ABH mouse strain may be a relevant EAE strain for investigating potential therapeutic treatments for MS disease.

6.1.2 Anti-CD4 antibody in EAE

CD4 is a marker found on a subset of T cells in the immune system. CD4 antibodies were extensively studied as a tool to modify the pathogenesis of EAE and gain a greater understanding of the inflammatory components of the disease. CD4-specific monoclonal antibody (mAb) suppress T cell dependent responses and have been shown to prevent the development of neurological EAE (O'Neill *et al.*, 1993). Administration of YTS191.1 (CD4-specific mAb) led to a rapid reduction of CD4+ T cells in the peripheral blood and lymphoid tissue by a complement mediated mechanism (Cobbold *et al.*, 1984). The basis of CD4-specific mAb immunomodulation is not completely understood but it is thought to prevent the function of activated effector cells (Sedgwick *et al.*, 1986). YTS177, a non-depleting CD4 mAb, results in the down regulation of CD4 and thus cannot cause interaction of CD4 with MHC class II antigens, which is required for T cell activation following engagement of the TCR with the MHC antigen complex (O'Neill *et al.*, 1993).

6.1.3 Anti-CD20 antibody in EAE

CD20 is a surface marker found on mature B cells and the marker is lost during differentiation into plasma cells. CD20 is not found on pre-B cells, these then differentiate into antibody producing cells.

Rituximab is a humanized mouse monoclonal antibody against the human CD20 surface molecule expressed by B cells. Rituximab® depletes a subpopulation of B cells (pre-B and mature B cells) without affecting antibody production and secretion (Petereit *et al.*, 2008). Rituximab® has been shown to reduce relapse rate by about 50% in relapsing-remitting MS (Bar-Or *et al.*, 2008).

In mice, it has been shown that Rituximab depletes B cells in the peripheral blood, secondary lymphoid organs and in the central nervous system (CNS). EAE severity was reduced in a transgenic EAE model treated with Rituximab prior to immunization or at the onset of the clinical signs (Monson *et al.*, 2011).

6.1.4 Mitoxantrone in EAE

Mitoxantrone is an anthracenedione which acts by intercalating into the DNA molecule, it also interferes with RNA, and uncoiling and repair of damaged DNA by inhibiting topoisomerase II enzyme (Fox, 2006). Mitoxantrone acts in a dose-dependent manner and is a potent immunosuppressive agent which inhibits in vivo induced proliferative response (Baker *et al.*, 1992). Clinical trials data suggest efficacy of mitoxantrone in reducing relapses and slowing disease progression in relapsing-remitting and secondary progressive MS (Debouverie *et al.*, 2007). A nonspecific cytotoxicity effect on lymphocytes is believed to be a major mechanism responsible for the clinical effects of mitoxantrone in MS patients.

Mitoxantrone causes a reduction in CD4, CD8 and B cells in the spleen (Piao *et al.*, 2007), and has been shown to significantly reduce the severity of acute phase EAE and also can prevent the onset of EAE if used pre-infection in a dose-dependent manner (Baker *et al.*, 1992).

6.2 Results

6.2.1 Anti-CD20 antibody depletes B cells in the blood and spleen

Depletion of B cells was investigated in the blood and spleen at various time points following a single i.v. treatment of naive unimmunized ABH mice with 250 μ g anti-CD20 antibody (section 2.3). The time points were days 7, 14 and 21 post immunization; blood and splenocytes were stained for B cell markers (B220) detailed in section 2.7.1. B cell percentages in spleen and blood were calculated (Figure 6.1 and 6.2). Reduction in B cells proportion was detected in the spleen and in blood up to day 21 post administration of anti-CD20. To ensure that B cells were maximally depleted before onset of EAE in the ABH mice, anti-CD20 antibody was given to the mice at least 3 days before the expected clinical signs of disease.

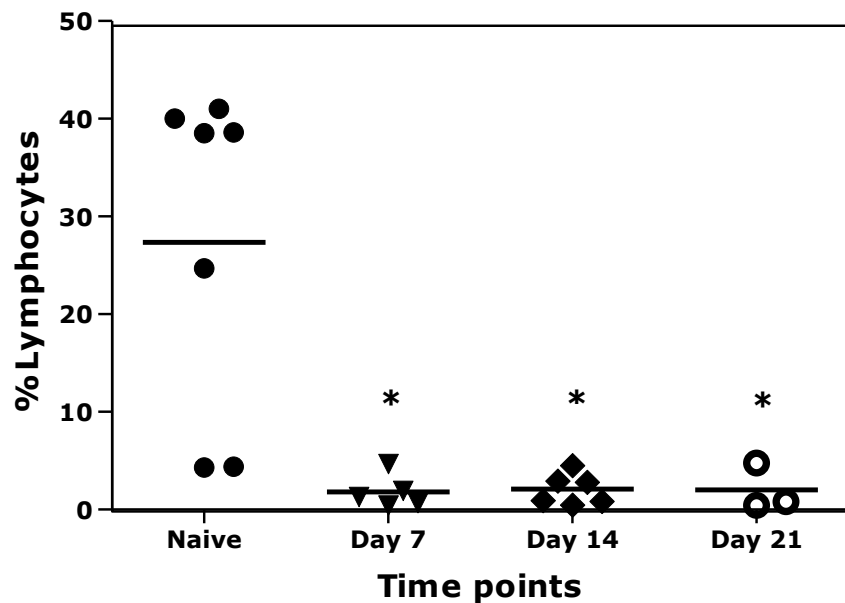


Figure 6.1: Percentage B cells in blood from ABH mice immunized intravenously with anti-CD20 antibody. Each mouse was given 250 μ g/ml anti-CD20 i.v. and cardiac bleeds taken on Days 7, 14 and 21 (n=7). The results show B cell percentages in lymphocyte population analysed. . *P<0.05 compared to naive.

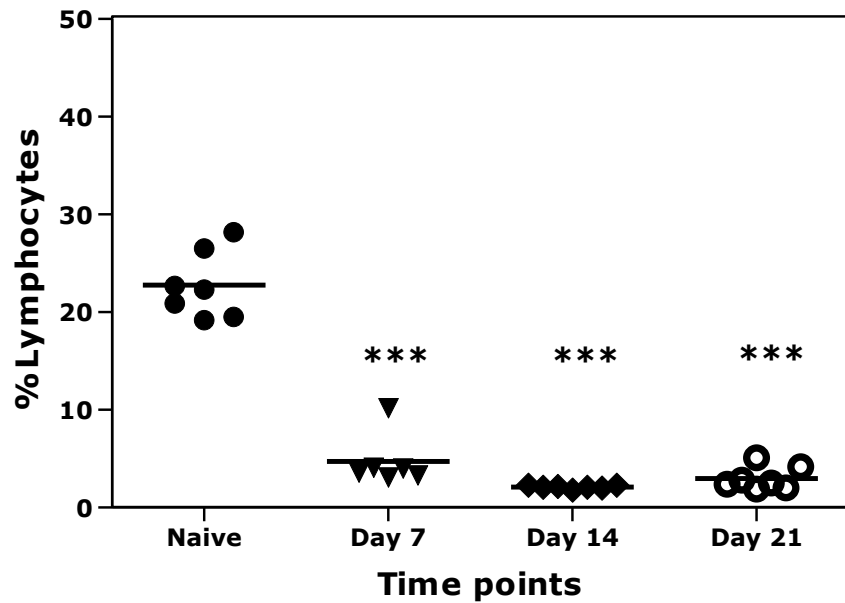


Figure 6.2: Percentage B cells in spleen of ABH mice immunized intravenously with anti-CD20 antibody. Each mouse was given 250 μ g/ml anti-CD20 i.v. and spleens taken on Days 1, 3, 7, 16 and 21 (n=7). Splenocytes were stained for B cell markers. The results show B cell percentages in lymphocyte population analysed. ***P<0.0005 compared to naive.

6.2.2 B cell proportions similar in anti-CD20 treated and untreated mice following EAE induction

B cell proportions were measured in terminal blood samples at the end of relapse-remitting EAE in the ABH mice. This was done to investigate the long term effect of anti-CD20 treatment on the B cell proportions. The treated group was given anti-CD20 antibody either at acute only or at the acute and relapse phases of the disease and the untreated group was used as a control (figure 6.3). Similar B cell proportions were observed in both the treated and untreated groups (figure 6.4).

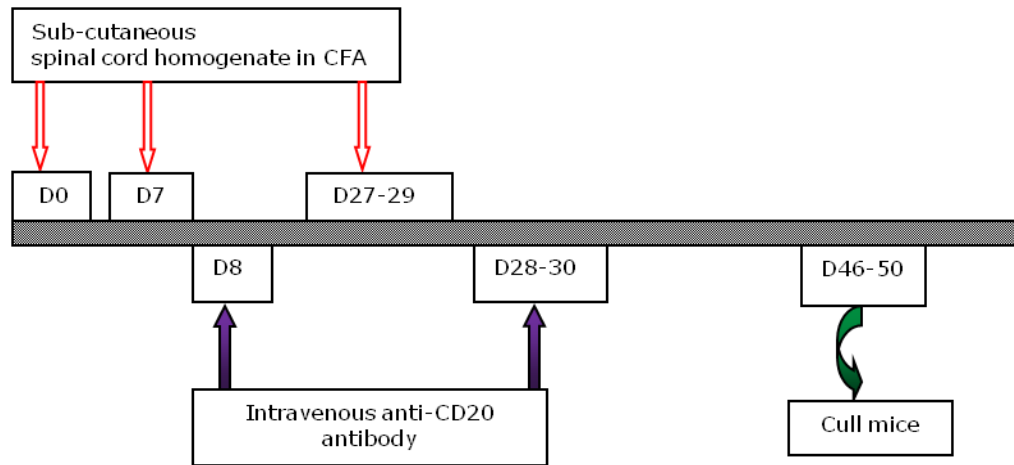


Figure 6.3: Timeline for immunization schedule for the administration of anti-CD20 antibody in EAE induced mice.

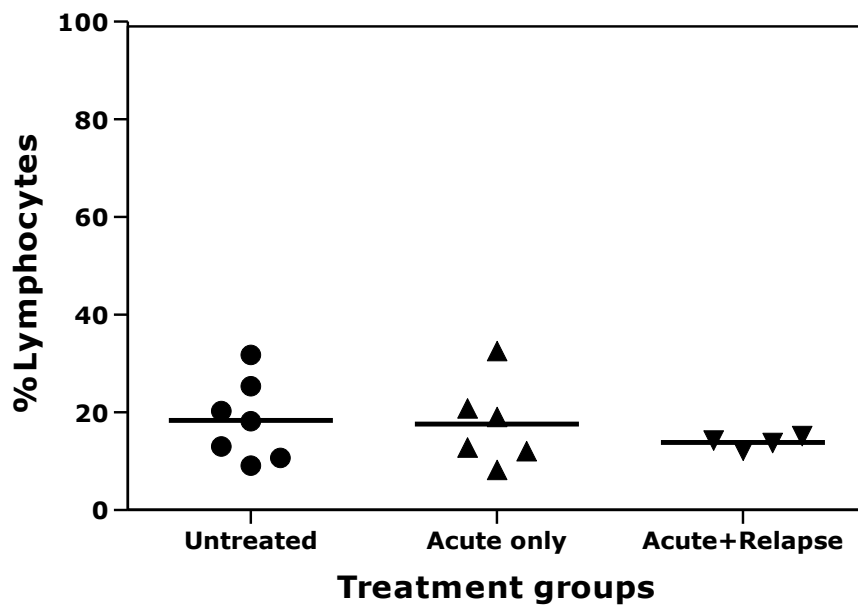


Figure 6.4: Percentage B cells in blood of ABH mice post relapse EAE. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7 and relapse induced on day 28. Mice were treated with 250µg/ml anti-CD20 i.v on day 8 (acute only group) and day 29 (acute+relapse group) and the control group was untreated. Terminal cardiac bleed were taken and cells stained for B cell markers. The results show B cell percentages in lymphocyte population analysed.

6.2.3 Acute anti-CD20 treatment does not alter EAE severity

Pre-treatment with anti-CD20 antibody has been shown to alter EAE severity in a recombinant mouse model (Monson *et al.*, 2011). The effect of anti-CD20 treatment on the severity of EAE was investigated in the ABH mouse. The mice were treated with 250 μ g anti-CD20 on day 8 prior to the onset of acute EAE (figure 6.3). In these mice, the severity of EAE was similar to those observed in the untreated group (figure 6.5). There was no significant differences between the untreated and anti-CD20 treated groups in the maximal EAE score day of onset of clinical signs or the number of mice with EAE (table 6.1).

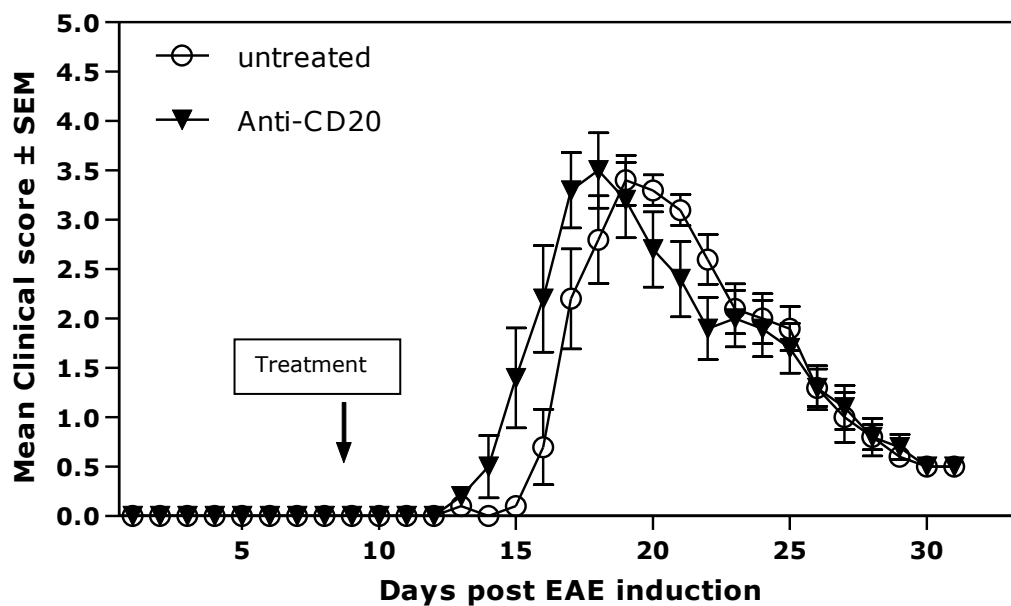


Figure 6.5: Treatment of relapsing remitting EAE with anti-CD20 antibody. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7. Animals were given 250 μ g/ml anti-CD20 antibody on day 8. The results represent the mean \pm SEM daily score (n=10).

Treatment	Dose	No. EAE/Total	Max EAE Score ±SEM	Day of onset ±SEM
Untreated	-	9/10	3.8 ± 0.3	17.7 ± 1.3
Anti-CD20	250 µg	9/10	3.9 ± 0.2	16.0 ± 1.1

Table 6.1: Treatment of relapsing remitting EAE with anti-CD20 antibody. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7. Animals were given 250 µg/mouse anti-CD20 antibody on day 8. The results represent the mean maximal score of animals that developed EAE, the number of animals that developed EAE and the day of onset of clinical signs.

6.2.4 Therapeutic anti-CD20 treatment does not alter EAE relapse

The effect of anti-CD20 treatment in established EAE was investigated. 250 µg of anti-CD20 was given to each mouse on day 29 following established EAE symptoms and prior to the onset of a relapse (figure 6.3). Severity of EAE in anti-CD20 treated group was similar to that observed in untreated group (figure 6.6). The maximal score and the number of animals that developed EAE were similar in the treated and control group. Although it was observed that therapeutic treatment with anti-CD20 did not appear to alter EAE relapse, the treatment led to a lengthening of the average day of onset of disease (table 6.2).

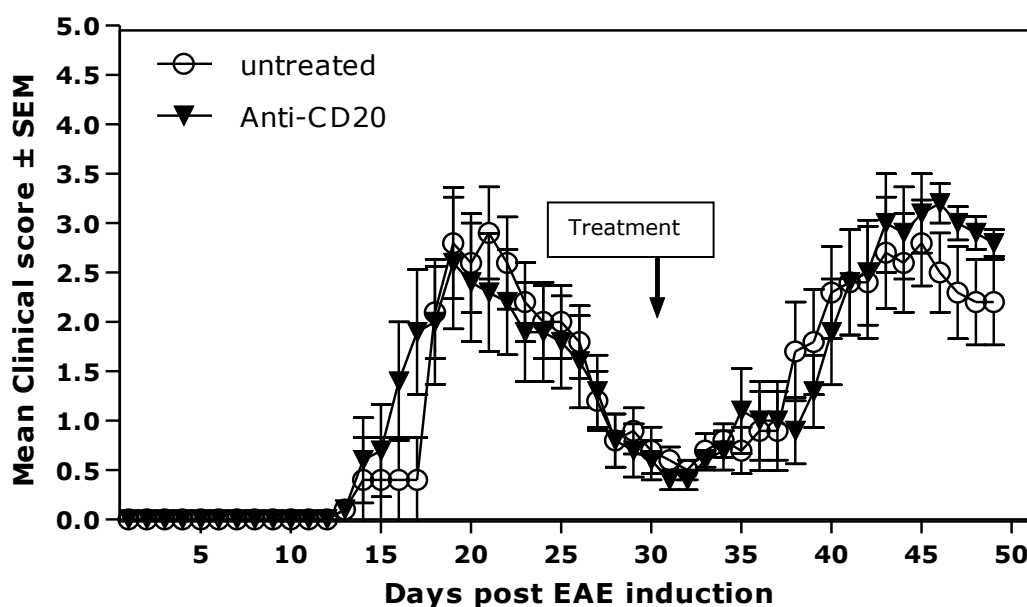


Figure 6.6: Treatment of relapsing remitting EAE with anti-CD20 antibody. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7, and a relapse induced on day 29. Animals were given 250 μ g/ml anti-CD20 antibody on day 28. The results represent the mean \pm SEM daily score (n=10).

Treatment	Dose	No. EAE/Total	Max EAE Score \pm SEM	Day of onset \pm SEM
Untreated	-	6/9	3.8 \pm 0.4	38.8 \pm 1.8
Anti-CD20	250 μ g	8/9	3.6 \pm 0.5	41.3 \pm 1.8

Table 6.2: Treatment of relapsing remitting EAE with anti-CD20 antibody. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7, and a relapse induced on day 29. Animals were given 250 μ g/mouse anti-CD20 antibody on day 28. The results represent the mean maximal score of animals that developed EAE and the day of onset of clinical signs in the relapse phase.

6.2.5 Multiple anti-CD20 treatment does not significantly alter EAE severity

Single treatment with anti-CD20 in established EAE pre-acute or pre-relapse did not alter the severity of EAE in the ABH model. The effect of multiple treatments with anti-CD20 in established EAE was explored. The mice were each given 250 μ g anti-CD20 antibody pre-acute and pre-relapse on days 8 and 28; and the control group was untreated (figure 6.3). There was essentially no reduction in severity of EAE in the treated group in comparison with the untreated group in both the acute and the relapse phases of the disease (figure 6.7). Likewise there was no significant reduction in the number of mice with EAE observed in the relapse phase in the treated group in comparison with the untreated group; similar maximal scores and day of onset of disease was observed in both groups (table 6.3).

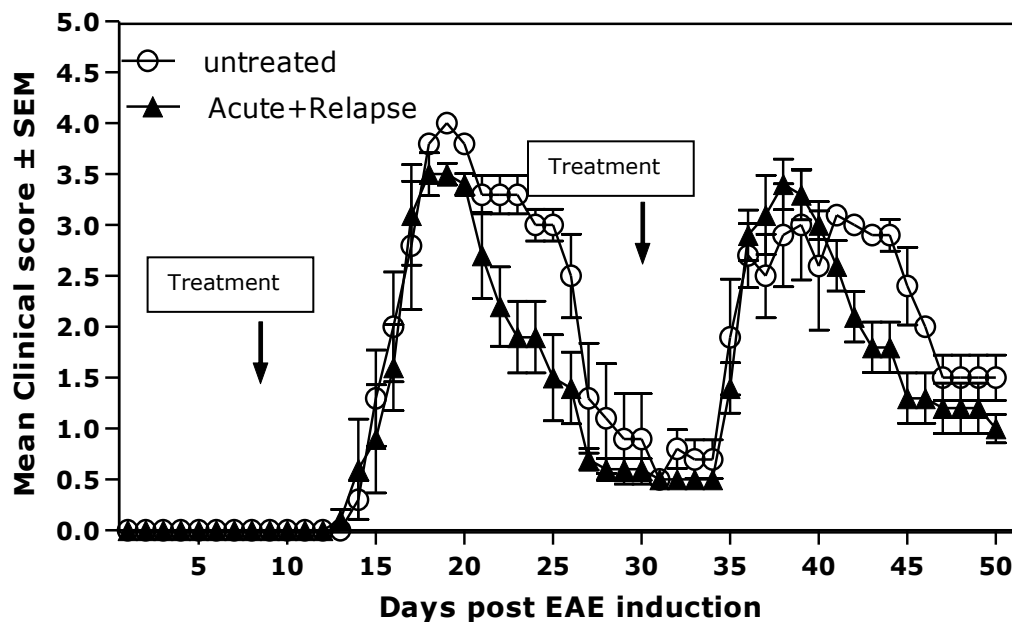


Figure 6.7: Treatment of relapsing remitting EAE with anti-CD20 antibody. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7, and a relapse induced on day 29. Animals were given 250 μ g/ml anti-CD20 antibody on days 8 and 28 (Acute+Relapse group) or untreated (untreated group). The results represent the mean \pm SEM daily score (n=8/9).

A

Treatment	Dose	No. EAE/Total	Max EAE Score ±SEM	Day of onset ±SEM
Untreated	-	9/9	3.8 ± 0.3	16.6 ± 0.7
Anti-CD20	250 µg	8/10	3.8 ± 0.6	18.4 ± 1.7

B

Treatment	Dose	No. EAE/Total	Max EAE Score ±SEM	Day of onset ±SEM
Untreated	-	7/7	3.8 ± 0.3	37.0 ± 2.4
Anti-CD20	250 µg	4/7	3.5 ± 0.4	36.5 ± 1.9

Table 6.3: Treatment of relapsing remitting EAE with anti-CD20 antibody. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7, and a relapse induced on days 29. Animals were given 250 µg/mouse anti-CD20 antibody on day 8 and 28. The results represent the mean maximal score of animals that developed EAE and the day of onset of clinical signs in the acute phase (A) and in the relapse phase (B).

6.2.6 Mitoxantrone and anti-CD4 antibody treatment alters EAE severity

Mitoxantrone and anti-CD4 antibody were given as positive controls as they had previously been shown to alter the severity of EAE in the EAE model (Baker *et al.*, 1992; Pryce *et al.*, 2005). Mitoxantrone and anti-CD4 antibodies were administered on day 8 pre-acute phase and both altered the severity of EAE in both the acute and the relapse phase (figure 6.8). There was a reduction on the number of animals with EAE in the mitoxantrone and anti-CD4 treated groups in comparison with the untreated group (table 6.4). A lengthening of the day of onset of disease was observed in the acute phase following anti-CD4 treatment.

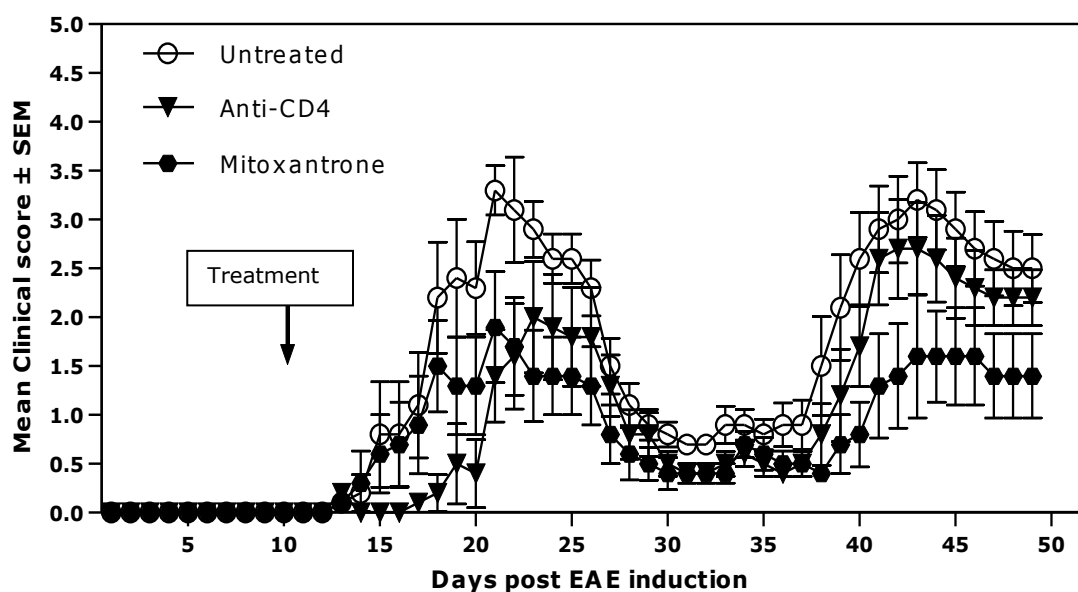


Figure 6.8: Treatment of relapsing remitting EAE with anti-CD4 antibody or mitoxantrone. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7, and a relapse induced on days 29. Animals were given 200 μ g/mouse anti-CD4 antibody or 2.5 mg/kg mitoxantrone on day 8. The results represent the mean \pm SEM daily score.

A

Treatment	Dose	No. EAE/Total	Max EAE Score ±SEM	Day of onset ±SEM
Untreated	-	8/10	3.9 ± 0.2	18.5 ± 1.9
Anti-CD4	200 µg	5/10	3.7 ± 0.3	21.8 ± 1.6
Mitoxantrone	2.5 mg/kg	4/9	3.9 ± 0.3	18.8 ± 2.6

B

Treatment	Dose	No. EAE/Total	Max EAE Score ±SEM	Day of onset ±SEM
Untreated	-	8/10	3.8 ± 0.4	39.5 ± 1.5
Anti-CD20	200 µg	7/10	3.7 ± 0.5	40.9 ± 1.1
Mitoxantrone	2.5 mg/kg	4/9	3.9 ± 0.3	41.8 ± 2.5

Table 6.4: Treatment of relapsing remitting EAE with anti-CD4 antibody or mitoxantrone. EAE was induced with spinal cord homogenate in complete Freund's adjuvant on days 0 and 7, and a relapse induced on days 29. Animals were given 200 µg/mouse anti-CD4 antibody or 2.5 mg/kg mitoxantrone on day 8. The results represent the mean maximal score of animals that developed EAE and the day of onset of clinical signs in the acute phase (A) and in the relapse phase (B).

6.3 Discussion

EAE is thought to be a T cell mediated disease, but recent results from clinical trials and EAE studies has led to the re-examination of the function/importance of B cells in the pathogenesis of EAE and MS (Qin *et al.*, 2001).

This chapter details the effects of B cell depletion by anti-CD20 antibody administration on the severity of EAE. Initial studies investigated the depletion of B cells by administering anti-CD20 antibodies in naïve ABH mice. Intravenous administration of anti-CD20 in naïve mice led to significant reduction in B cells in the blood and a reduction in the spleen. 3 days post immunization showed the lowest levels of B cells in the blood and the levels were back up 2 weeks post immunization. As a result of the preliminary studies, anti-CD20 was administered at least 1-3 days before the onset of clinical disease to investigate the involvement of B cells in the development of clinical signs of EAE. In a spontaneous relapsing-remitting EAE model, it was shown that depletion of B cells by administration of anti-CD20 antibody suppressed RR-EAE when treatment was started at a young age (Pollinger *et al.*, 2009). Anti-CD4 depleting antibodies have been shown to prevent the development of EAE (O'Neill *et al.*, 1993) and Mitoxantrone has also been shown to reduce severity and onset of EAE disease (Baker *et al.*, 1992). Anti-CD20, anti-CD4 and mitoxantrone were used in this study because they are the depleting strategies that were used in the NAb model studied (discussed in chapters 4 and 5). These were used to investigate their effect on the immune system in the EAE setting which is relevant to MS disease.

Previous studies using different EAE models and different EAE induction methods have shown varying contradictory effects of anti-CD20 antibody administration on the disease. In a C57BL/6 human CD20 transgenic mouse model, it was observed

that administration of anti-CD20 before EAE induction with MOG₃₅₋₅₅ or after onset of EAE led to increased disease severity (Weber *et al.*, 2010). On the contrary in another study, it was observed that administration of anti-CD20 at the peak of EAE disease led to rapid recovery and reduced cumulative disease in the C57BL/6 mice (Matsushita *et al.*, 2008). In the C57BL/6 mice, B cell depletion before EAE induction led to no change in disease onset but disease was more severe in these MOG₃₅₋₅₅ EAE induced mice (Matsushita *et al.*, 2008). When anti-CD20 antibody was administered in these mice before EAE onset, there was no change in disease as was observed in this study. In this study, anti-CD20 antibody was injected intravenously 1-3 days before the onset of clinical signs of EAE and there was no significant reduction in severity of the disease and also no significant effect on the onset of disease. On the other hand, in my project when anti-CD20 was given multiple times (pre-acute and pre-relapse, there was a modest reduction in the severity of relapse disease. This study shows that B cells were depleted by anti-CD20 antibody and this may be important in the severity of EAE disease especially in the relapse phase of the disease in the Biozzi ABH model. Post relapse phase, the proportion of B cells in spleen in both the treated and untreated groups were similar, signifying that the B cell levels in the treated group had increased post depletion.

This study supports the use of anti-CD20 depletion of activated B cells in the treatment of EAE and by extrapolation MS. Anti-CD20 depletes antibody generating B cells and these may be relevant in the neutralizing antibody model studied in chapter 3. Selective depletion of B cells may be a valuable strategy to further improve efficacy of B cell targeted therapies in MS and other inflammatory CNS demyelinating diseases.

Chapter 7

General discussion and conclusions

7.1 Key findings

This project met the main aim to develop and establish a neutralizing antibody mouse model to recombinant human interferon beta (rhIFN β). Initial studies involved 2 mouse models Biozzi ABH and BALB/c mice as potential NAbs models. Preliminary research showed that NAbs were more consistently detected in BALB/c mice than in ABH mice. The immunization protocol for establishing of NAbs was developed in the BALB/c mice using Betaferon®. RhIFN β -specific T cell responses (proliferation and cytokine production) were observed in the established NAbs model. Depletion strategies were developed in naïve BALB/c mice, anti-CD4 and anti-CD20 antibodies significantly depleted CD4 T cells and B cells respectively in the blood and spleen following administration. Mitoxantrone induced toxicity in animals and so did not appear useful to examine the effects of Mitoxantrone in tolerance at the doses tested. These depletion strategies were used in conjunction with intravenous administration of rhIFN β to induce tolerance in the NAbs model. I.v. administration of rhIFN β in the NAbs model resulted in a reduction in the incidence of NAbs by 10-20%; the incidence of NAbs was further reduced in combination with depletion strategies. RhIFN β -specific CD4 and CD8 T cell responses were reduced by the induction of tolerance in this project.

BALB/c mice were used to develop the NAbs model in my project. The mice were immunized with recombinant human interferon beta (rhIFN β) which is seen as a foreign protein by the mice. The amino acid sequences are different in human and in mice though there are many similar sequences within the protein. As a result of

the differences, the mice mounted an immune response to rhIFN β when immunized with the protein. Neutralizing antibodies to the protein were detected, and antigen-specific IFN γ production was also detected in my project. NAbs have previously been detected in BALB/c mice to cytomegalovirus (Fang *et al.*, 2014), and to rhIFN β (Rebif and Avonex) (Bellomi *et al.*, 2007) using Rebif and Avonex. Antibodies to Betaferon has also previously been detected in BALB/c mice (Jiskoot *et al.*, 2013). In my project Betaferon was used as this has previously been shown to be more immunogenic than the other 2 forms of rhIFN β and NAbs to the protein were detected. The NAbs model was developed and used in studying tolerance induction to rhIFN β . A human IFN β transgenic model was developed in the C57/BL6 mice to study tolerance (Hermeling *et al.*, 2005). The mechanism of tolerance was by breaking of tolerance as the mice recognized rhIFN β as a self-protein. In my project on the other hand, the mechanism of tolerance induction was the mice recognized rhIFN β as a foreign protein. In preliminary studies, CD4 and D8 T cells mounted an antigen-specific response to rhIFN β by cytokine production (IFN γ and IL17) as early as day 7. Further immunization led to an expansion of these effector cells. Proliferation of CD4 T cells were also enhanced by subcutaneous immunization with rhIFN β . Initial responders require a 2 signals to initiate a response but memory cells have been shown to require less stringent stimulation to initiate or sustain a memory response as was shown in in my project in the proliferation and IFN γ production observed in memory CD4 and CD8 T cell populations.

Mitoxantrone, anti-CCD4 and anti-CD20 antibodies were the choice for the depletion strategy in my project as these have been trialed or are approved for the treatment of MS. Anti-CD4 and anti-CD20 antibodies significantly reduced CD4 T cells and B cells respectively in the spleen and blood in naive BALB/c mice. Mitoxantrone reduced CD4 T cells and B cells in the blood and significantly reduced CD4 and CD8 T cells in the spleen, there was also a significant reduction in macrophages and

monocytes. Macrophages are microbicidal, antigen-presenting and cytokine-producing cells and play a crucial role in resistance to pathogens. These cells may play a role in development of disease (Sinha *et al.*, 2008), as was observed in development of EAE symptoms in my project. There was a reduction in EAE symptoms in mice that were immunized with mitoxantrone (which significantly reduced monocytes and macrophages) in comparison with the control group that were not immunized. Macrophages as APCs could be involved in NAb production, a reduction could lead to reduction in NAb production. Due to the toxicity of mitoxantrone, this avenue could not be studied further in this project.

Intravenous administration of antigen have been shown to induce tolerance. Induction of immune tolerance was successfully achieved in patients with hemophilia A with NAb to recombinant factor VIII (FVIII) using repeated i.v. FVIII which resulted in reduction of NAb in majority of patients (Astermark *et al.*, 2006). Single i.v. IFN β administration in MS patients with NAb to the drug has been shown to decrease NAb in the short-term (Millonig *et al.*, 2009). However in a pilot study, continuous high-dose i.v. IFN β did not appear to induce tolerance to IFN β , but established its bioactivity in MS patients with high NAb titres (Skrobald *et al.*, 2014). Tolerance has been induced in mice via the intravenous route of immunization. In my project, i.v. administration of rhIFN β in naïve BALB/c mice led to induction of tolerance as observed by the reduction in CD4 T cell proliferation in response to the antigen. In established NAb model, i.v. administration did not appear to affect the outcome of NAb incidence. It is most likely that prior immunization with rhIFN β results in the development of mature rhIFN β -specific B cell and intravenous administration of rhIFN β leads to relocation of the antigen to the spleen where the primed B cells can rapidly respond. In a mouse model, it was observed that single administration of rhIFN β led to NAb production and i.v. administration led to increased levels of NAb which suggest a booster effect on

primed cells as observed after vaccine administration (Kijanka *et al.*, 2013). The route of administration of antigen is vital in determining the response observed. It is believed that subcutaneous administration is the most immunogenic and i.v. is the least immunogenic. However, it has been observed in some studies that i.v. administration of anti-TNF α for instance is more immunogenic when injected i.v. than when administered subcut in the cynomolgus monkey (Emery 2003). In my project i.v. administration of IFN β in naïve BALB/c led to reduced NAbs incidence.

7.2 Value of the NAbs mouse model

The NAbs mouse model is a valuable model for studying different strategies for induction of tolerance to rhIFN β . The main advantage of the NAbs model is that the mechanism of NAbs induction is similar to that observed in IFN β -treated MS patients with NAbs; IFN β is seen as a foreign protein and an immune response is mounted. This model can also be used to study aspects of NAbs development, elimination and control.

Preliminary immunization protocols showed that NAbs was induced and was observed with an incidence 50-70% after day 35. RhIFN β -specific CD4 and CD8 T cells proliferative responses were also observed in this model. Once NAbs was established in this model, then tolerization strategies were investigated and developed in the model and NAbs incidence and CD4/CD8 T cell responses were used as indicators for tolerance induction. In this model, intravenous administration of rhIFN β in the NAbs model led to a reduction in the incidence of NAbs and also a reduction in CD4/CD8 T cell responses to the protein. Depleting cells prior to tolerance induction further reduced the incidence of NAbs. The NAbs mouse model do not show any physical signs once NAbs has been induced and this is an

advantage over the EAE model (mouse model for MS) where neurological signs are observed. The results observed shows that the NAbs model could potentially be used to investigate and develop a better understanding of NAbs development and tolerance induction strategies.

As a mouse model, there are limitations that need to be looked at closely. The most significant limitation of the model is incidence of NAbs which is between 50-70%, therefore reducing the number available for each study. To overcome this limitation, the immunization protocol can be further developed to increase the incidence of NAbs in the mouse model. In this project different immunization schedules were investigated and the best one used for the project which involved immunizing sub-cut on days 0, 7 and 21 with 20 μ g Betaferon in CFA. Other rhIFN β formulation could also be used in developing the immunization strategy. Another strategy could be the use of other bacteria within the CFA to boost the response to IFN β . Another weakness is the volume of samples that can be obtained from these animals at any time point. Due to the small volume of blood or plasma obtained, the development of assays and the number of assay used for investigating tolerance induction was limited. One way to overcome this would be to pool samples in groups and thereby increasing sample volumes.

NAbs in the context of IFN β (NAbs model) was chosen rather than MS directly. Establishing of the NAbs mouse model has several implications for work in the field of research into neutralizing antibodies in MS patients with NAbs to IFN β . The mechanism of NAbs development in both the model and in MS patients are similar, it is hoped that strategies used in the NAbs model can be extrapolated for use in MS patients. Intravenous administration of IFN β in combination with depletion strategies reduced the incidence of NAbs in this model, and further methods of

tolerance induction can be investigated in this model. NAbs to IFN β in MS patients have been shown to reduce the bioavailability of the drug (Francis et al., 2005); induction of tolerance to IFN β would therefore help in eliminating NAbs and increasing the bioavailability of the drug.

7.3 Future work

Chapter 3: Establishing of NAbs model.

- (a) Further develop the immunization schedule (including use of different IFN β formulations) in the NAbs model to increase the incidence of NAbs.
- (b) Investigate the development of a NAbs model in larger animals such as the rat, to increase sample volumes for assay development, optimization and analysis.

Chapter 4: Development of depletion strategies

- (a) Investigate the use of other available depleting antibodies in the NAbs model, including anti-CD52 (alemtuzumab) and anti-CD19 antibodies.

Chapter 5: Development of tolerization strategy

- (a) Further develop the induction of tolerance by intravenous rhIFN β , the volume to be used and the timings for re-introduction of the protein.

Chapter 6: EAE and depletion strategy

- (a) The depletion strategies to be developed in chapter 4 above (anti-CD52 and anti-CD19) can be tested in the EAE model.

7.4 Conclusions

This project successfully met the aim to develop a neutralizing antibody “NAbs” mouse model against recombinant human interferon beta for the study of tolerance induction strategies, which is relevant for IFN β -treated multiple sclerosis patients that have developed neutralizing antibodies.

1. Neutralizing antibodies were successfully developed in the BALB/c mice. An immunization protocol was developed and refined resulting in more consistent establishment of NAbs to rhIFN β .
2. Depletion strategies using CD4+ and CD20+ depleting antibodies and mitoxantrone successfully deleted cell subsets in naïve BALB/c mice.
3. Tolerance successfully induced in NAbs model by intravenous administration of rhIFN β .
4. Tolerance enhanced by depletion of cell groups prior to intravenous re-introduction of rhIFN β being the best strategy.
5. Depletion strategies employed in the EAE setting successfully reduced the incidence of EAE.

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